

Exploring Probiotics-Host Interactions

Intestinal Immune and Defence Responses to
Lactobacillus reuteri in Health and Disease

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2017

Acta Universitatis agriculturae Sueciae

2017:40

Cover: Probiotics-host interactions
(photo: Shokoufeh Karimi)

ISSN 1652-6880

ISBN (print version) 978-91-576-8853-8

ISBN (electronic version) 978-91-576-8854-5

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Print: SLU Service/Repro, Uppsala 2017

Exploring Probiotics-Host Interactions – Intestinal Immune and Defence Responses to *Lactobacillus reuteri* in Health and Disease

Abstract

Lactobacillus reuteri, a ubiquitous inhabitant of the mammalian gastrointestinal (GI) tract has known health-promoting effects and various strains are commercially available as probiotics. Several probiosis mechanisms have been suggested in *L. reuteri*'s mode of action, but the mediators and factors involved are not well understood. This thesis examined the function of probiotics, particularly *L. reuteri*, in the GI tract by equipping *L. reuteri* ATCC PTA 6475 and R2LC with reporter genes (luminescence and fluorescence) and a mutant of strain 6475 was generated by inactivation of chaperon *dnaK*. Different *in vitro* and *in vivo* applications of fluorescent and luminescent strains were evaluated, and it was demonstrated that flow cytometry can be a powerful method for determination of plasmid persistence. Biophotonic imaging (BPI) enabled low doses ($\sim 1 \times 10^5$) of luminescent bacteria to be monitored in the GI tract and revealed retention of large numbers of bacteria in the stomach up to 3 hours post-gavage. The effect of four strains of *L. reuteri* (6475, R2LC, DSM 17938, 1563F) was examined in an epithelial infection model using IPEC-J2 cells induced by enterotoxigenic *E. coli*. By analysing transepithelial electrical resistance (TEER) and leakage of FITC-dextran, it was shown that *L. reuteri* pre-treatment prevented damage by ETEC to epithelial monolayer integrity. The strains also reduced expression of pro-inflammatory cytokines (TNF α and IL-6) and maintained expression of adherens junction (E-cadherin) and upregulated tight junction (ZO-1) proteins.

To further explore the *L. reuteri* mode of action, five mutants were evaluated in DSS-induced acute colitis and IPEC-J2 models. It was found that *dnaK*⁻, *pduC*⁻, *cmbA*⁻, amidase⁻ and *srtA*⁻ may not play major roles in the mechanisms by which 6475 maintains mucosal integrity and counteracts inflammation. However, mutants 6475 *pduC*⁻ and 6475 *cmbA*⁻ had a tendency to weaken the protective effect of 6475 in the colitis model.

Studies on the effects of *L. reuteri* strains on mast cell activation and inflammatory response revealed no inhibition of degranulation mediated by IgE-antigen activation, but downregulated expression of the pro-inflammatory cytokines IL-6 and IL-13, irrespective of degranulation. Thus pre-treatment with *L. reuteri* strains can protect against intestinal barrier dysfunction and mucosal inflammation, partly through altering junctional complex proteins, mediators of immunity and mast cells.

Keywords: mucosal barrier, *Lactobacillus reuteri*, biophotonic imaging, pro-inflammatory cytokines, adherens junction, tight junction, IPEC-J2, colitis, mast cells

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Dedication

To my family.

It is not the strongest of the species that survive nor the most intelligent, but the one most responsive to the changes.

Charles Darwin, 1809

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Karimi, S*, Ahl, D., Vågesjö, E., Holm, L., Phillipson, M., Jonsson, H. & Roos, S. (2016). *In vivo* and *in vitro* detection of luminescent and fluorescent *Lactobacillus reuteri* and application of red fluorescent mCherry for assessing plasmid persistence. *PLoS One* 11(3): e0151969.
- II Shokoufeh Karimi, Torbjörn Lundh, Hans Jonsson, Stefan Roos. *Lactobacillus reuteri* strains protect the epithelial barrier integrity of IPEC-J2 monolayers from the detrimental effect of enterotoxigenic *Escherichia coli* (ETEC) (*Manuscript*).
- III Shokoufeh Karimi, David Ahl, Lena Holm, Mia Phillipson, Hans Jonsson, Stefan Roos. Exploring *Lactobacillus reuteri* probiosis mechanisms in DSS-induced colitis and *in vitro* infection models (*Manuscript*).

A preliminary report on interactions between mast cells and *L. reuteri* is also included in the thesis.

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* Corresponding author. Shokoufeh Karimi

The contribution of Shokoufeh Karimi to the papers included in this thesis was as follows:

- I Participated in planning the study. Performed the majority of the lab work. Main writer of the manuscript.
- II Participated in planning the study. Performed the majority of the lab work. Main writer of the manuscript.
- III Participated in planning the study. Performed the majority of the lab work. Main writer of the manuscript.

For preliminary study report: Participated in planning the study. Performed the majority of the lab work. Main writer of the report.

Abbreviations

CCL	Chemokine (C-C motif) ligand
CmbA	Cell- and mucus binding protein A
DC	Dendritic cell
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FCM	Flow cytometry
FcεR	High-affinity IgE receptor
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
Ig	Immunoglobulin
IL	Interleukin
IPEC	Intestinal porcine epithelial cell
ITAM	Immunoreceptor tyrosine-based activation motif
LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
NAD	Nicotinamide adenine dinucleotide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PC	Plate count
PCMC	Peritoneal cell-derived mast cell
PRR	Pattern recognition receptor
SCFA	Short chain fatty acid
ssp.	Subspecies
TEER	Transepithelial electrical resistance
TGFβ	Transforming growth factor β

Th	T helper
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
ZO	Zonula occludens

1 Introduction

1.1 General background

The human body is constantly exposed to different types of microorganisms present in the environment. The first line of host defence in the early phase is provided by physical barriers such as skin and mucus in different parts of the human body. The inner wall of the intestine is a specialised barrier against food-derived antigens and microbes. The human gastrointestinal (GI) tract is a complex and dynamic ecosystem harbouring trillions of microbes known as the microbiota. This master organ serves as a major regulator of human and animal health through digestion and defence and immunity. It is believed that the GI tract skilfully communicates with other organs in the human body and regulates their function. In fact, the intestine could be considered the largest immune organ, as it is highly involved in tolerance and immunity. The interaction of the mucosa with lumen content (*i.e.* food and microbiota) preliminarily defines the microbial community. A disturbance in the composition and function of the microbiota (dysbiosis) has been associated with hyperpermeability of the intestine (leaky gut) and, as a result, pathogenesis of many disorders and diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), diabetes type I and many more.

The intestinal microbial community in humans is dominated by the phyla Firmicutes and Bacteroidetes. *Lactobacillus* species belong to the Firmicutes and are considered beneficial members of the GI tract. Several *Lactobacillus* species have been shown to have beneficial effects on human and animal health, and are currently permitted for use as probiotics.

Elie Metchnikoff (1845-1916) can be considered the father of the probiotic concept. Although, the term ‘probiotics’ was not introduced by him, he was the first to describe the benefits of administering sour milk containing “Bulgarian

Bacillus” (later described as *Lactobacillus delbrueckii* subsp. *Bulgaricus*). Use of probiotics was born when physicians started prescribing the sour milk diet for patients. The term probiotics, which was introduced by Lilley and Stillwell (1965), is nowadays part of our common vocabulary.

Awareness of the importance of *Lactobacillus* has led to use of this microorganism in the food/feed industry and recently in the medical and healthcare fields. *Lactobacillus reuteri* is ubiquitously indigenous to the GI tract of mammals. Protection and restoration of the gut barrier and function by *Lactobacillus reuteri* has previously been reported in animal models and human clinical trials, but the exact mechanisms of their probiosis are not yet known. The possibilities of probiotic therapies have been assessed for promoting health and curing diseases in human and animals for several decades (Iannitti and Palmieri, 2010). Today, probiotics are available in food and dietary supplements (offered as capsules, tablets, powders or liquid suspensions) that each contain a specific type of probiotic bacteria or mixture of a few probiotic strains.

This thesis investigated the probiotic mechanisms of action of *L. reuteri*. The thesis starts with an introductory part, providing a broad background to the physiology of the GI tract, gut immunity and defence, including mucosal barrier, probiotics and an overview of mechanisms of action of probiotics, with the focus on *L. reuteri* strains and their health benefits. **Chapter 2** lists the aims of the work. The material and methods used to conduct these studies are then briefly described in (**Chapter 3**), followed by a summary of the results (**Chapter 4**) and a discussion (**Chapter 5**).

This thesis also presents a preliminary study on the interaction between mast cells and *L. reuteri* and the effect of probiotics on mast cell activation, which gives further knowledge regarding the *L. reuteri*-host immune response. The methods used to perform this study are described in detail in (**Chapter 3**) and the results and a discussion of the findings at the end of (**Chapter 4**) and (**Chapter 5**), respectively.

1.2 Gastrointestinal (GI) tract physiology and defence mechanisms

1.2.1 The gastrointestinal (GI) tract

The gastrointestinal (GI) tract is a hollow tube that extends from mouth to anus. The small and large intestine are two main parts of the GI tract. The small intestine is divided into three different anatomical and physiological sections (duodenum, jejunum and ileum) and is the major site for the absorption of

nutrients, water and minerals (Volk and Lacy, 2017). In humans, the lower part of the GI tract is called the large intestine or colon. It is 90-150 cm long, is located immediately after the small intestine and consists of ascending, transverse and descending loops and the rectum, which ends with the anus. The large intestine is also involved in the absorption of water and electrolytes (Kararli, 1995, Mowat and Agace, 2014).

1.2.2 The intestine wall

The wall of the GI tract has four basic layers: mucosa, muscularis mucosa, submucosa and serosa (Figure 1). The inner layer, the mucosa, has two main functions; secretion and absorption and being a selective barrier. Therefore, maintenance of the mucosal functions is extremely important. The mucosa in the small intestine is folded to form crypts and villi, which increase the area of the active epithelium and absorption, while villi are absent from the colon (Johansson et al., 2011a, Mowat and Agace, 2014). The intestinal epithelial cells (IEC) (enterocytes) of the mucosa are joined tightly with neighbouring cells by tight junctions and are covered by a mucus layer.

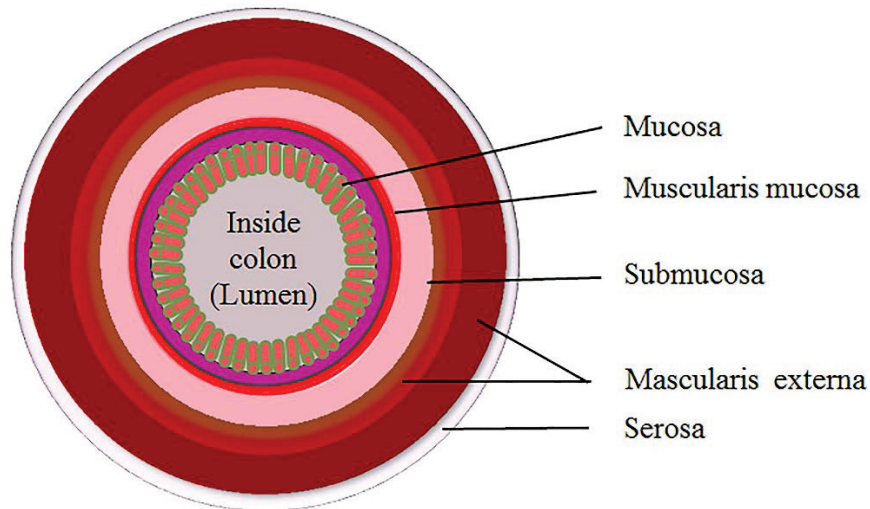


Figure 1. The wall of the gastrointestinal tract and its four different layers: mucosa, muscularis mucosa, submucosa and serosa.

1.2.3 Intestinal barriers

The intestinal barrier and gut permeability

The main function of the GI tract is the digestion and absorption of nutrients and electrolytes. However, it has another extremely important function; regulating the host-environment interaction through its barrier function. The disruption of the mucosal barrier leads to translocation of luminal antigens and gut microbes into the lower layers of the intestine.

Intestinal permeability is associated with the barrier function and is defined as “the facility with which enterocytes allow molecules to pass through by non-mediated passive diffusion” (Travis and Menzies, 1992). This property of the intestinal mucosa is associated with passage of ions and molecules of low molecular weight across the gut wall. Two different pathways, paracellular and transcellular pathways, are involved in molecular transport from the lumen to the lamina propria, and are facilitated by junctional complexes between neighbouring intestinal epithelial cells and transcytosis, respectively (Menard et al., 2010). The intestinal barrier function is maintained by a complex multilayer system and can be divided into outer physical and inner immunological barriers (Figure 2). Disruption of the intestinal barrier, followed by infusion of luminal antigens, induces the mucosal immune response and inflammation, which is known to be a cause of onset and development of many intestinal and systemic diseases (Lee, 2015).

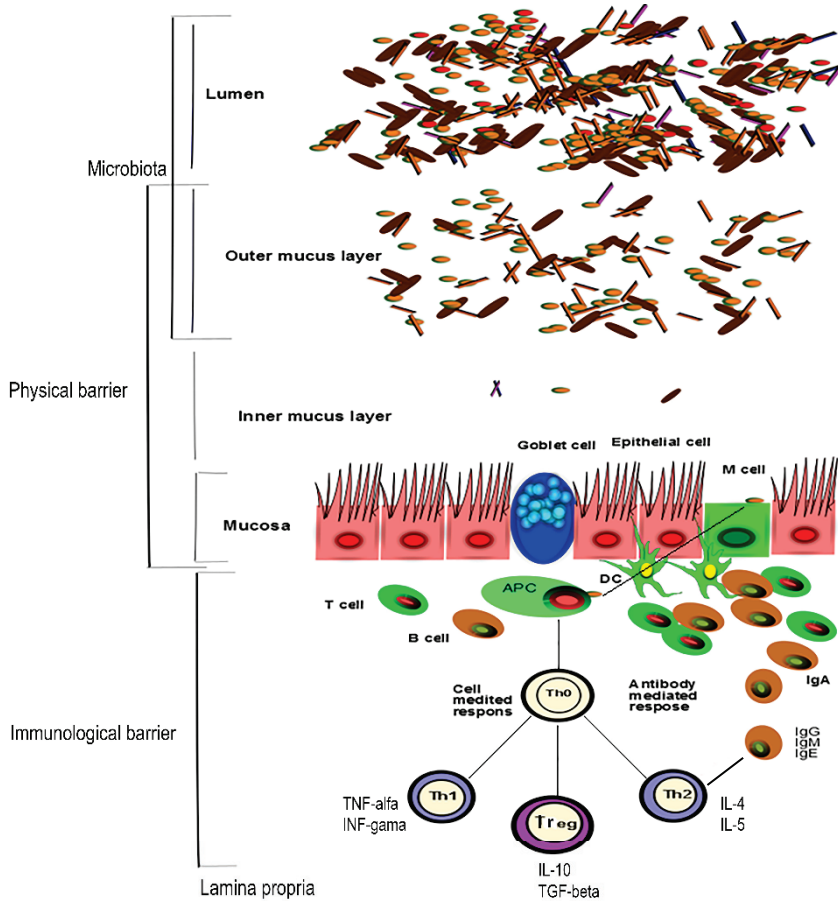


Figure 2. The different layers of intestinal barrier: inner and outer mucus layers, a single layer of intestinal epithelial cells and an immune barrier in the lamina propria.

Outer physical barrier

The intestinal barrier is a major line of defence between the body and the external environment, with a surface area of 300-400 m² (Anderson et al., 2012). The outer physical barrier of the intestine is made up of a single layer of columnar epithelial cells covered by a mucus layer.

(i) Mucus layer

The mucus layer has direct contact with food, microbes and antigens in the GI tract (Mowat and Agace, 2014). It is also considered to be a chemical barrier in

the intestine and has a thickness that varies from 10 to 100-200 μm in different parts of the intestine (Lundquist and Artursson, 2016). The mucus is secreted by goblet cells and is mainly composed of mucins, which are glycosylated proteins and have an ability to filter molecules with a cut-off size of 600-800 Daltons. The mucin in the colon and small intestine is mainly MUC2 (Wang et al., 2005).

The mucus layer of the small intestine consists of one layer, while that in the large intestine has two layers (Johansson et al., 2011b, Antoni et al., 2014). The mucus layer in the small intestine is not continuous and is less well defined compared with the colon with its thick inner firmly adhering and outer loosely adhering mucus layers (DeSesso and Jacobson, 2001, Atuma et al., 2001). The loosely adhering mucus layer is a major habitat for commensal bacteria and the firmly adhering inner layer largely has a protective function against luminal bacteria (Johansson et al., 2011b, Johansson et al., 2008b, Dicksved et al., 2012).

The loosely adhering layer is considered to be produced as a result of proteolytic cleavage of MUC2 by the proteolytic enzyme meprin β (Johansson et al., 2008a, Birchenough et al., 2015). The mucus layer is continuously renewed by the goblet cells, which decelerate penetration by microbes (Birchenough et al., 2015).

(ii) *Intestinal epithelial cells*

Around 80% of the intestinal epithelial cells (IEC) on the villi surface are columnar absorptive cells (*i.e.* enterocytes) (Van der Flier and Clevers, 2009). In the crypts of the villi, there are stem cells that continually divide and differentiate into different cell types, including absorptive enterocytes, enteric endocrine cells, mucus-secreting goblet cells, Paneth cells that secrete antimicrobial peptides and microfold (M) cells that sample the antigens. Apart from Paneth cells, which remain in the crypts, the other cell types migrate up to the tips of the villi in order to replace the aged cells at the tips (DeSesso and Jacobson, 2001, Menard et al., 2010). Epithelial barrier unity and polarity are maintained by the junctional complexes. The duties of these junctions are enabling passage of small macromolecules, electrolytes and fluids, and inhibition of passage of large macromolecules through the barrier (Lee, 2015).

(iii) *Cellular components of the mucosal barrier*

Junctional complexes are vital for cell-cell contact and can be divided into three different structures: tight junctions, adherens junctions and desmosomes (Zihni et al., 2016) (Figure 3).

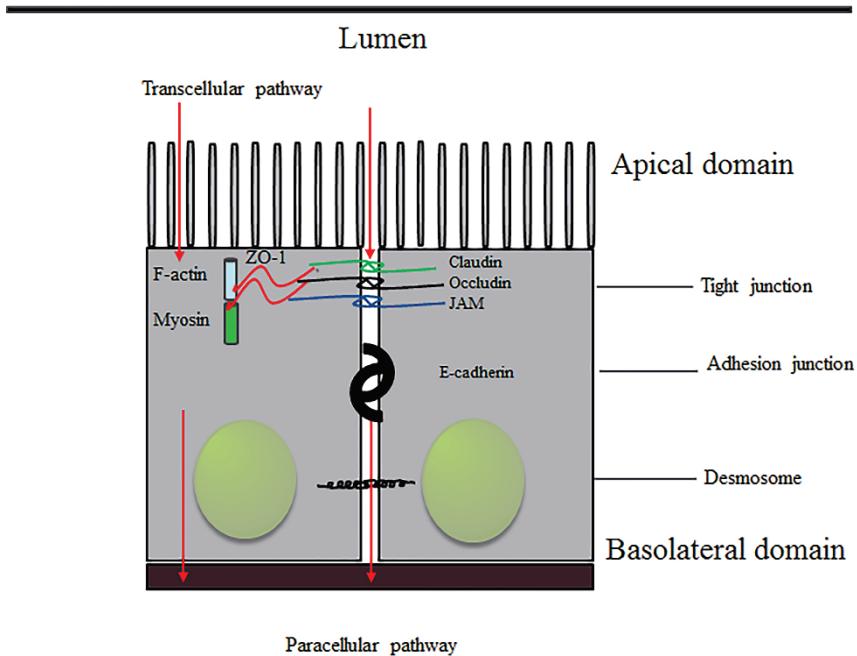


Figure 3. Schematic diagram of junctional complexes, showing the location of tight junctions (TJ), adherens junctions (AJ) and desmosomes in epithelial cells.

Epithelial tight junctions (TJs) are mainly responsible for regulating paracellular influx of different sizes of molecules between the epithelial cells. Tight junctions are multi-protein complexes, including both transmembrane and peripheral membrane proteins. They interact with the cytoplasmic cytoskeleton in the cytoplasm and interact with the neighbouring cells. The claudin family and zonula occludens are the most important transmembrane and peripheral membrane proteins, respectively, and have crucial roles in assembly of the junction barriers. Intracellular domains of the transmembrane proteins interact with intracellular scaffolding proteins, zonula occludens and actin cytoskeletons (Suzuki, 2013, Zihni et al., 2016). The tight junction barriers can be divided into the paracellular barrier, with selective permeability for both large and small molecules through different diffusion pathways, and the intramembrane barrier, which restricts the exchange between the apical and basolateral cell surfaces (Zihni et al., 2016).

Adhesion junctions and desmosomes are involved in communication and adhesion between the cells that maintain cellular proximity. Adherens junctions are composed of a family of transmembrane proteins, cadherins including E-cadherin and catenin, that contributes to development of the perijunctional

actomyosin ring (Suzuki, 2013). Desmosomes are hyper-adhesive proteins of intercellular junctions and are involved in cell-cell communication and bindings (Garrod and Chidgey, 2008).

Inner immunological barrier and defence in the GI tract

(i) Response to antigenic bodies and microbes

When a pathogen crosses the physical barrier, it is exposed to the first line of immune defence, the innate immune system. This part of the immune system plays an essential role in the early recognition of pathogens (Mogensen, 2009) and is mostly facilitated by antigen-presenting cells (APCs) and phagocytic cells (Iwasaki and Medzhitov, 2004). The host innate immune response is initiated upon recognition of conserved structures of microbes known as pathogen-associated molecular patterns (PAMPs) (Van den Abbeele et al., 2011) or microorganism-associated molecular patterns (MAMPs) (Lin and Zhang, 2017) through pattern recognition receptors (PRRs), including the families of toll-like receptors (TLRs) and nucleotide-like receptors (NLRs). Upon exposure to the pathogen, PAMPs are recognised by the PRRs present at the cell surface and pro-inflammatory and antimicrobial responses are activated via induction of a multitude of signal transduction pathways (Akira and Takeda, 2004). Subsequently, gene expression is activated and numerous molecules, including cytokines, chemokines and cell adhesion molecules, are synthesised, which together organise the early and late phase infection, including induction of the adaptive immune response (Akira et al., 2006). Therefore, invading microbes that escape the innate defence encounter the second line of defence, an activated adaptive response.

The gut constitutes the largest immune organ of the body due to the presence of the gut-associated lymphatic tissue (GALT). The GALT is divided into organised tissues consisting of Peyer's patches, mesenteric lymph nodes (MLNs) and smaller isolated lymphoid follicles that are involved in the induction phase of the immune response. The Peyer's patches are lymphoid nodules in the submucosa of the small intestine that are covered by a follicle-associated epithelium (FAE) and characterised by the presence of M cells that are directly involved in antigen sampling from the lumen and delivering it to antigen-presenting cells such as dendritic cells (DC) (Geboes, 2003, Ohno, 2016). The processed antigen is introduced to immune cells by antigen-presenting cells and antigen-specific effector cells that migrate to the mesenteric lymph nodes (MLN) and, as a result, further mucosal immunity or tolerance is full-fledged. Furthermore, based on the type of stimulus, naive B cells can undergo immunoglobulin class switching from expression of IgM to IgA (Mowat, 2003).

Antigen translocation is controlled by IgA. The IgA-antigen complex can also be recognised by IgA receptors on the M cells. In the lamina propria, antigen-presenting dendritic cells receive the antigen, which is then processed and presented to T cells. As a result, T cells are primed and differentiated into different T cell types (Th/Treg) based on the antigenic immune-stimulation and expressed cytokines from APC and other cells (Anderson et al., 2012).

(ii) *Immune mediators*

Cytokines are small peptides or proteins that are produced by various cell populations such as immune and epithelial cells, and are involved in cell signalling. Cytokines may act on the secreting cell (autocrine action), on nearby cells (paracrine action) or on distant cells (endocrine action) (Zhang and An, 2007). They play a central role in coordinating maturation of the immune system and regulating responses to antigens, but also by activating several functions of epithelial cells, which in response produce many cytokines and chemokines that further regulate the immune responses (Lunney, 1998, Vilček, 2003, Scheller et al., 2011).

(iii) *Some examples of cytokines*

TNF- α is a pro-inflammatory cytokine and a master regulator of pro- and anti-inflammatory cytokines with a diverse range of biological activities. This multifunctional cytokine affects lipid metabolism, coagulation, insulin resistance and endothelial (Stenvinkel et al., 2005) and epithelial dysfunction (Al-Sadi et al., 2009). *TNF- α* is produced by various cell types, including monocytes, macrophages, B cells, T cells, and NK cells, in response to different stimuli such as bacterial toxins and inflammatory products (Tracey and Cerami, 1993).

IL-13 is a multifunctional cytokine (Minty et al., 1993) and is produced by, and affects, many cell types such as macrophages, mast cells, epithelial cells and fibroblasts (Wynn, 2003). It is produced in high levels by CD4⁺ Th2 cells, and in lower levels by Th0 and CD8⁺ T cells (de Waal Malefyt et al., 1995). IL-13 is functionally associated with IL-4 and, although the cytokines share several functions, IL-13 appears to play a more essential role than IL-4 (Wynn, 2003). Several effector functions are known for IL-13, including regulation of gastrointestinal parasite expulsion, airway hyperresponsiveness (AHR), allergic inflammation, mastocytosis, IgE and antibody production, goblet cell hyperplasia and mucus production, tissue remodelling and fibrosis (Wynn, 2003).

IL-6 is a cytokine with both pro- and anti-inflammatory properties (Scheller et al., 2011) and is produced by various types of immune cells, including fibroblasts, adipocytes, mesothelial, monocytes and lymphocytes. IL-6 is often produced in response to stimuli, such as bacterial endotoxins, oxidative stress physical exercise, TNF- α and IL-1 β (Stenvinkel et al., 2005).

(iv) *Mast cells*

Mast cells are a versatile cell type and are part of the innate immunity (Meurer et al., 2016). They are highly granulated leukocytes that are derived from haematopoietic stem cells. Following migration from bone marrow, they enter the blood circulation as progenitor cells and undergo final differentiation and maturation. They are usually present in high numbers in vascular tissues that encounter the external environment, such as skin, intestine and airways. Upon exposure and early recognition of antigens, mast cells are activated and undergo degranulation, which leads to the release of a variety of pre-formed mediators such as histamine, the proteases tryptase and chymase, and large numbers of cytokines and chemokines (Abraham and St John, 2010, Dawicki and Marshall, 2007).

Mast cells are able to interact directly with PAMPs through expression of TLRs. Mast cells are known for their involvement in allergies and the best studied mechanism for the activation and degranulation of mast cells is through stimulation of high-affinity IgE receptor Fc ϵ RI (Abraham and St John, 2010, Gilfillan and Tkaczyk, 2006). However, degranulation and activation can also occur in response to other external stimuli such as complements, neuropeptides and certain toxins (Wernersson and Pejler, 2014).

An allergic reaction is established when APCs encounter an allergen and as a result B cells produce antigen-specific immunoglobulin E (IgE). The IgE molecules bind to Fc ϵ RI on the surfaces of mast cells and, after re-encountering the same allergen, cross-linking of Fc ϵ RI-associated IgE molecules by the antigen leads to activation of the mast cell. Fc ϵ RI receptor consist of three different subunits; α -, β - and two γ -chains that can form a trimeric $\alpha\gamma_2$ or tetrameric $\alpha\beta\gamma_2$ structure on the cell surfaces. The α -chain possesses high affinity for the Fc region, whereas the β - and γ -chains are involved in signal transduction through immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylation of the ITAMs by the Lyn protein leads to activation of spleen tyrosine kinase (Syk), which phosphorylates several signalling molecules, leading to degranulation (Garman et al., 2000, Turner and Kinet, 1999).

1.3 Microbiota of the GI tract and inflammatory bowel disease

1.3.1 The gut microbiota

The complex microbial community in the GI tract, which is known as the gut microbiota, consists of bacteria, fungi, archaea and viruses (Marchesi et al., 2016). A recent investigation estimated the number of bacterial cells in the gut microbiota to be higher than 10^{13} , which is 10-fold greater than the total number of human cells (Sender et al., 2016).

The gut contains more than 1000 species, including eukarya (~100), archaea (~10) and bacteria (~1000) (Rajilić-Stojanović and de Vos, 2014). The colon contains the largest population of microorganisms in the GI tract, 10^{11} bacteria per gram. The stomach and the upper part of the small intestine contain 10^3 to 10^4 bacteria per g and the lower small intestine 10^8 bacteria per g (Sender et al., 2016). The most diverse and abundant bacteria belong to the four phyla Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria (Rajilić-Stojanović and de Vos, 2014). Degradation of complex plant-derived polysaccharides and fermentation of carbohydrates are the main activities of the gut microbiota. The microbiota is known for its beneficial effect on the host and is considered to be an intestinal barrier that has a constructive impact on epithelial proliferation, survival and metabolism (Neish, 2009). In addition, as a result of fermentation of carbohydrates, the microbiota produces metabolites like short-chain fatty acids (SCFA), including acetate, propionate and butyrate (Kien, 1996, Marchesi et al., 2016) that affect intestinal physiology and immune functions (Maynard et al., 2012, Canani et al., 2011). Butyrate is considered to be the most important SCFA for intestinal epithelial cell proliferation and differentiation, villi development, mucin production, intestinal motility, defence mechanism and barrier function of the gut (Guilloteau et al., 2010, Hamer et al., 2008, Havenaar, 2011). The microbiota of the GI tract also enables its role of regulating the microbial community through limiting pathogen colonisation by competing for adhesion sites, increasing mucin production, producing antimicrobial compounds and optimising the composition of the microbiota (Neish, 2009).

A healthy intestinal microbiota maintains intestinal immune homeostasis. Changes in microbial composition, function or local distribution are termed dysbiosis (DeGruttola et al., 2016). Dysbiosis can occur as a result of several factors, such as infection, ingestion of antibiotics and a poor diet (Budden et al., 2017, Delzenne et al., 2011). Dysbiosis can lead to several intestinal and systemic diseases and disorders (DeGruttola et al., 2016), such as inflammatory

bowel disease (Manichanh et al., 2006, Peterson et al., 2008), type 2 diabetes (Karlsson et al., 2013) and obesity (Turnbaugh et al., 2006, Ley et al., 2006).

1.3.2 Inflammatory bowel disease (IBD)

Intestinal barrier dysfunction has been implicated in the pathophysiology of many intestinal diseases, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), coeliac disease and many more (Konig et al., 2016, Peterson et al., 2008).

Inflammatory bowel disease is a group of chronic relapsing disorders of the GI tract that includes ulcerative colitis (UC) and Crohn's disease (CD). Inflammatory bowel disease is common in developed countries and the prevalence of the disease is 1 out of 200 individuals in the northern European countries (Gismera and Aladren, 2008). Ulcerative colitis is usually superficial, starts from the rectum and spreads proximally toward the colon. Crohn's disease causes segmental transmural inflammation that can affect the entire GI tract, although the lower part of the ileum and proximal colon are the most affected sites (Geboes, 2003).

The exact aetiology and pathogenesis of IBD is currently unknown, but several studies have shown strong evidence of a contribution of dysregulated immune responses, especially overly aggressive cell-mediated immunity to, in particular, the intestinal microbiota in genetically susceptible individuals suffering from a gut barrier dysfunction (Sartor, 2006a, Konig et al., 2016).

The microbiota of IBD patients has been shown to differ from that of healthy individuals. It is not known whether the changes in the microbiota are the cause or consequence of the inflammation, but a balance between the microbiota and immune response seems to be a prerequisite for a healthy intestinal barrier (Vindigni et al., 2016).

Strong support for involvement of the intestinal microbiota in IBD is provided by reports of therapeutic effects of antibiotics on dampening the flare-up of the disease and an effect of probiotics that can counteract the symptoms of intestinal damage and inflammation, and ameliorate IBD (Gionchetti et al., 2003, Sutherland et al., 1991, Oliva et al., 2012). In addition, data from a study using a mice model have revealed that presence of bacteria in the GI tract is required to initiate IBD, since IL-10-deficient mice developed colitis when colonised by enteric bacteria, but not under germ-free conditions (Hoffmann et al., 2011). Furthermore, genetic studies in humans and mice have shown that specific genetic variants of PRRs, which are involved in host-microbe interactions, have implications for the development of IBD (Inohara et al., 2003, Hisamatsu et al., 2003, De Jager et al., 2007, Fukata and Arditi, 2013). An

important aspect in the pathogenesis of IBD is dysregulation of the immune response to commensal bacteria in the intestine and it has been shown that establishment of Th1/Th2 and Th17/Treg cell imbalances is implicated in the pathogenesis of IBD (Sun et al., 2015).

1.4 Probiotics

1.4.1 Introduction to probiotics

Health-promoting benefits of consumption of microorganisms, especially lactic acid bacteria (LAB), a group of Gram-positive bacteria that are commonly used in food fermentation, have been known for many years. In 1965, the term ‘probiotic’ was used by Lilly and Stillwell to describe substances produced by protozoa that could stimulate the growth of other organisms (Kaur et al., 2002). In 2001, probiotics were defined by the World Health Organization (WHO) as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO, 2001). Probiotics comprise both bacteria and yeasts and the most commonly used probiotic bacteria belong to *Lactobacillus* and *Bifidobacterium*.

1.4.2 Selection criteria for probiotics

Selection criteria for a probiotic may include: 1) Stability of phenotype and genotype; 2) proven functional properties of the probiotic strain compared with the original isolate (Forssten et al., 2011, Ouwehand and Lahtinen, 2008); 3) acid and bile resistance, and survival and growth during gastrointestinal transit (Dunne et al., 2001); probiotics do not always need to colonise the intestinal tract to exert their effects (Ohland and MacNaughton, 2010), but they need to persist and be alive; 4) excluding or reducing pathogenic adherence; 5) capable of producing acids, hydrogen peroxide and bacteriocins antagonistic to pathogen growth (Kaur et al., 2002); 6) not having any transferable antibiotic resistance (Panel, 2012); 7) being safe, non-pathogenic, non-allergenic, non-mutagenic/carcinogenic and capable of being tolerated by the immune system and not provoking immunity (Desai, 2008)

1.4.3 Mechanism of action of probiotics

Evidence-based analyses of data from human clinical trials have shown the clinical potential of probiotics against diseases and disorders (Indrio et al., 2014, Oliva et al., 2012, Harata et al., 2016). Mechanisms of probiotic action are

versatile and their beneficial effects are not a reflection of a single mechanism. Differences in mechanism not only exist among species, but may also exist between strains within the same species. The beneficial impact of probiotics often shows a discrepancy in results from experimental and clinical trials (Gou et al., 2014, Kristensen et al., 2016, Farnworth, 2008, Boyle et al., 2009, Huang and Huang, 2016, Sanders et al., 2013). This could be explained by differences in the models and techniques, types of probiotics and in *in vivo* studies, because of complexity of the signalling network in the GI tract, including probiotics-host and probiotics-intestinal microbiota interactions and treatment strategies. In addition, communication with the host is not restricted to the intestine, but extends to distant locations and the complicated network of the gut-organ axis.

Probiotics may exhibit their health-promoting benefit through three main modes of action: competitive exclusion, improvement of barrier function and immune modulation.

Competitive exclusion along the epithelium

Several studies have shown adhesion of probiotics to the intestinal mucosa *in vitro* (Boudeau et al., 2003, Resta-Lenert and Barrett, 2003, He et al., 2001) and *in vivo* (Valeur et al., 2004). It has been claimed that this adhesion of probiotics to the intestinal mucosa limits nutrient availability to other bacteria and restricts pathogen access to the epithelium (O'Hara and Shanahan, 2007). The adhesion of probiotics is mediated by several adhesion factors (Buck et al., 2005, van Pijkeren et al., 2006). The attachment of probiotics to the intestinal mucosa is mainly mediated by proteins. Some examples of adhesion proteins are mucus-binding protein (MUB) produced by *Lactobacillus reuteri* (Roos and Jonsson, 2002, Buck et al., 2005), and CmbA (cell- and mucus-binding protein A), which is reported to mediate the binding of *L. reuteri* to the mucus (Jensen et al., 2014). Furthermore, probiotics may induce expression of mucins and subsequent thickening of the mucous layer that covers the intestinal mucosa (Ahl et al., 2016, Caballero-Franco et al., 2007), which as a result may inhibit invasion by pathogens (Mack et al., 1999, Mack et al., 2003, Mattar et al., 2002). In addition, probiotic bacteria can modify the composition of the lumen microenvironment and inhibit pathogen growth through the production and/or release of a variety of antimicrobial factors. These antimicrobial factors include bacteriocins, hydrogen peroxide, nitric oxide, lactic and acetic acids and de-conjugated bile acids (Oelschlaeger, 2010b).

Enhancement of epithelial barrier function

The intestinal barrier is the major defence mechanism of the host against the surrounding environment and disruption of the barrier causes penetration by bacterial and food antigens, which induce inflammatory responses (Sartor, 2006b). The mechanisms by which probiotics enhance intestinal barrier function are not fully understood. Studies using animal and cell culture models have shown that probiotics can upregulate genes of the tight junction complex, for example E-cadherin and β -catenin (Anderson et al., 2010). Other studies have indicated that probiotics may initiate repair of the intestinal barrier function after damage through upregulation and redistribution of tight junction proteins ZO-2, ZO-1 and occludin (Zyrek et al., 2007, Yang et al., 2015b, Ahl et al., 2016). It has also been shown that probiotics can prevent pro-inflammatory cytokine-induced epithelial damage (Sartor, 2006b, Oliva et al., 2012, Kumar et al., 2017).

Furthermore, probiotic bacteria can prevent apoptosis in epithelial cells through regulation of both anti- and pro-apoptotic signalling pathways (Yan and Polk, 2002). Probiotics also induce mucus production in order to improve barrier function (Gaudier et al., 2005, Ahl et al., 2016).

Recently, a systematic literature review of human trials studying the effect of probiotic products on the intestinal barrier was performed (van Hemert et al., 2013). This review, of 29 published papers, found that the target patients, probiotic features and methods of measuring barrier function varied considerably. In approximately half of the studies reviewed, probiotics had positive effects and different markers of intestinal integrity such as zonulin, intestinal fatty acid-binding protein (IFABP), C-reactive protein (CRP), TNF- α , α -antitrypsin, lactulose/mannitol (L/M) test were assessed. It was concluded that the dosage, duration, bacterial strain and choice of marker for measuring intestinal integrity are important for future studies.

Immune modulation

It is well known that bacteria can influence the host immune system by their metabolites, cell wall components and DNA. They have the ability to interact with the intestinal epithelium, antigen-presenting cells like DCs and macrophages, and lymphocytes (Oelschlaeger, 2010a). The normal microbiota and possibly some probiotics create a tolerance state mediated by TLR7 and TLR3 on dendritic cells. However, the anti-inflammatory effects of probiotics may be mediated through the action of TLR9 signalling (Gomez-Llorente et al., 2010, Bermudez-Brito et al., 2012).

The signals from the microbiota are necessary for normal mucosal and immune development (Fukata et al., 2005, Rakoff-Nahoum et al., 2004). The

interaction is facilitated by PRRs (e.g. TLR) expressed on cells like enterocytes, dendritic cells and M cells (O'Hara and Shanahan, 2006), and initiates activation of signalling cascades of transcription of pro-inflammatory factors in immunosensory cells. TLRs play a crucial role in analysis of the microenvironment, which leads to discrimination between pathogens and the normal microbiota, while DCs can directly sample bacteria and prime the T cells, resulting in balancing of the T helper (Th) and regulatory T cell responses in the mucosa.

It has been shown that the modulation of DC maturation and cytokine expression is not the same for all probiotic strains (Christensen et al., 2002, Hart et al., 2004). It is also known that DCs from different lymphoid tissues exhibit different cytokine responses to probiotic and pathogenic bacteria (O'Mahony et al., 2006). Data from studies on animal models of diseases indicate that the therapeutic effects of probiotics could be mediated through downregulation of the pro-inflammatory cytokines TNF α and INF γ and enhancement of regulatory cytokines, such as TGF- β (McCarthy et al., 2003, Sheil et al., 2006, Madsen et al., 2001, Mohamadzadeh et al., 2005). Some *Lactobacillus* species, e.g. strains of *L. casei* and *L. reuteri*, adjust the immune response toward a tolerance state by activating the DCs to initiate the development of regulatory T cells with high expression of IL-10 and suppressing the proliferation of other effector T cells (Mohamadzadeh et al., 2005, Smits et al., 2005). Apart from modulation of T cell-mediated responses, probiotics have also been shown to enhance innate and humoral immune responses such as increased proportion and activity of phagocytic and natural killer cells, as well as levels of IgA in clinical trials (Olivares et al., 2010, Fang et al., 2000, Sheih et al., 2001, Rautava et al., 2006).

1.4.4 Health benefits of probiotics

Probiotic therapies have been evaluated for improvement of health and for curing diseases in human and animals for several decades (Iannitti and Palmieri, 2010). Evidence-based analyses of data from human trials and animal models have shown the clinical potential of probiotics against many diseases and disorders (Indrio et al., 2014, Oliva et al., 2012, Harata et al., 2016).

Some recent studies on the beneficial effect of probiotics have shown that some strains of probiotics can suppress diarrhoea caused by antibiotic treatment (Ruszczyński et al., 2008, Guarino et al., 2009) and *Clostridium difficile* (Plummer et al., 2010); improve lipid profiles and lower serum cholesterol (LDL and HDL levels) (Harata et al., 2016) subsequently reduce the risk of hypertension (Lye et al., 2009); reduce lactose intolerance (Vonk et al., 2012), and treat and prevent the recurrence of urogenital infections (Zuccotti et al.,

2008, Czaja et al., 2007). Probiotics can also have beneficial effects on atopic/allergic diseases (de LeBlanc et al., 2007), rheumatoid arthritis (Mandel et al., 2010), postoperative complications (Nomura et al., 2006), IBS (O'Mahony et al., 2005, Kajander et al., 2008) and IBD (Mikov et al., 2014).

1.5 *Lactobacillus reuteri*

Lactobacillus reuteri is a rod-shaped Gram-positive bacteria belonging to the genus *Lactobacillus*, which is the largest genus included in the LAB (Shahani and Ayebo, 1980). *Lactobacillus reuteri* was first described as a species in 1980 and was previously regarded as *Lactobacillus fermentum* biotype II (Kandler et al., 1980). It can colonise the digestive tract of humans and other mammals and birds (Casas and Dobrogosz, 2000), but is predominantly found in the gut of rodents, pigs and poultry (Walter et al., 2011). It has also been isolated from the human mammary duct (milk) and genital tract (discharge) (Abrahamsson et al., 2009, Reuter, 2001). *Lactobacillus reuteri* has been detected in only 10% of individuals (Valeur et al., 2004), but the proportion can vary depending on diet and population. For instance, a study in Papua New Guinea found 100% prevalence in that population (Martínez et al.). Analysis of faeces from healthy humans has indicated that the *Lactobacillus* genus accounts for less than 2% of total faecal bacteria (Sghir et al., 2000).

1.5.1 Importance of probiotic characteristics of *L. reuteri*

There are important characteristics of *L. reuteri* that make several strains of this species interesting as potential probiotics.

Lactobacillus reuteri has been successfully administered orally and has been shown to be able to tolerate and survive the acidic conditions of the GI tract and persist in the presence of bile salts in the small intestine, where its strong adherence to the mucus layer and intestinal epithelial cells has been demonstrated by several *in vitro* studies (Liu et al., 2014, Jensen et al., 2014, Walsham et al., 2016). This might be a requirement for the suppressive effect on pathogens seen in clinical trials (Savino et al., 2015).

Other important probiotic characteristics of *L. reuteri* are production of bioactive metabolites and compounds. It has been shown that strains of *L. reuteri* convert a dietary component, L-histidine, into histamine, which exerts anti-inflammatory effects (Thomas et al., 2012). Bacterial histamine downregulates expression of TNF- α via activation of the histamine receptor H2 (Thomas et al., 2012, Vannier et al., 1991).

Lactobacillus reuteri strains produce anti-microbial substances such as reuterin or reutericyclin. Reutericyclin is a cyclical tetramic acid and antibiotic produced by only some strains of *L. reuteri* (Holtzel et al., 2000). This metabolite disturbs the membrane of Gram-positive bacteria (Cherian et al., 2014) such as *Clostridium difficile* (Hurdle et al., 2011) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Hurdle et al., 2009), but not of Gram-negative bacteria and yeasts (Ganzle et al., 2000). Reuterin (3-hydroxypropionaldehyde) is another anti-microbial molecule that induces oxidative stress in microbes (Chung et al., 1989). Reuterin is formed during anaerobic growth as a result of glycerol dehydration. Reduction of reuterin to 1, 3-propanediol regenerates NAD⁺ from NADH, contributing to better growth (Schaefer et al., 2010, Chung et al., 1989).

It is known that not all *L. reuteri* strains are able to produce reuterin and that the level of reuterin production varies from one strain to another. For example, strains ATCC PTA 6475 and ATCC PTA 5289 produce lower levels than strains ATCC 55730 and CF48-3A (Jones and Versalovic, 2009).

Vitamin B12 is another important metabolite, production of which is linked to the capacity to convert glycerol to reuterin. The genes required for B12 synthesis have been identified in strains ATCC PTA 6475 (Santos et al., 2011) and JCM 1112 (Santos et al., 2011, Morita et al., 2008). However, the only report of B12 production by *L. reuteri* has been identified as pseudo vitamin B12 (Santos et al., 2007) and is produced by *L. reuteri* CRL 1098 (Santos et al., 2008, Taranto et al., 2003).

1.5.2 Mode of action of *L. reuteri* and intestinal health benefits

Lactobacillus reuteri has been shown to have positive effects on gastrointestinal barrier functions and the gut immune response, but the mechanism of action is not well known. However, it appears that *L. reuteri* exerts its beneficial effects through similar mechanisms as described for other probiotics including:

1. Regulating and re-directing the immune response toward an anti-inflammatory state and tolerance, through activation of T_{reg}, upregulation of anti-inflammatory cytokines and downregulation of pro-inflammatory cytokines (Oliva et al., 2012, Lin et al., 2008b, Ahl et al., 2016, Liu et al., 2013).
2. Improvement of mucosal barrier and permeability through upregulating tight junction proteins, increasing the thickness of the mucus layer (Ahl et al., 2016), upregulating defensins (Mackos et al., 2013) and regulating immune responses leading to tightening of the junctions between the cells, in particular apical junction complex (Yang et al., 2015b).

3. Exclusion of the pathogens by competing for adherence to the mucosa and mucus layer, as shown by several *in vitro* studies (Walsham et al., 2016, De Weirtdt et al., 2012) and in an infant clinical trial (Savino et al., 2015).
4. Re-modelling and correction of the microbiota in humans and animals with dysbiosis, as reported recently in several studies (McFarland, 2014, Marin et al., 2017, Buffington et al., 2016). In two animal studies, remodelling of microbiota by *L. reuteri* led to a decrease in multi-organ inflammation in scurfy mice (He et al., 2017) and correction of the oxytocin levels and subsequent defective social behaviour associated with neurodevelopmental disorders in offspring caused by a maternal high-fat diet during pregnancy (Buffington et al., 2016). In a recent study in a chronically stressed mice model, alteration of microbiota composition in particular reduced *Lactobacillus* and increased circulating kynurenine levels (Marin et al., 2017). It proved possible to re-establish the intestinal *Lactobacillus* levels by administering *L. reuteri*, which was sufficient to improve the metabolic shifts and despair behaviour in the mice model (Marin et al., 2017).

1.5.3 Some recent preclinical and clinical effects of *L. reuteri* on the gut

Preclinical studies

Several studies using animal models and cell cultures have shown an anti-inflammatory effect of *L. reuteri* (Thomas et al., 2012, Lin et al., 2008b, Jones et al., 2011, Thomas et al., 2016, Ahl et al., 2016). It has also been shown that oral pre-treatment with a cocktail of *L. reuteri* strains prevents onset of colitis in rats by downregulation of P-selectin and decreasing Crohn's disease leukocyte- and platelet-endothelial cell interactions (Schreiber et al., 2009). Another study using the same cocktail of *L. reuteri* strains (R2LC, JCM 5869, ATCC PTA 4659 and ATCC 55730) demonstrated that *L. reuteri* does not improve the disruption of the mucus barrier caused by dextran sulphate sodium (DSS), but can reduce the DSS-associated bacterial translocation to mesenteric lymph nodes (Dicksved et al., 2012). A study Crohn's disease using *L. reuteri* R2LC has also demonstrated that intracolonic administration prevents the development of acetic acid-induced colitis in rats and normalise Crohn's disease the myeloperoxidase (MPO) activity and mucosal permeability (Fabia et al., 1993). Moreover, it has been shown that oral administration of *L. reuteri* I5007 in newborn piglets improves mucosal integrity and upregulates expression of the apical junction proteins claudin-1, occludin and zonula occluden-1 (ZO-1) (Yang et al., 2015b).

In an *in vitro* study using the IPEC-J2 cell line, it was shown that pre-treatment with a *L. reuteri* strain isolated from porcine gut prevented disruption of the mucosal integrity caused by enterotoxigenic *Escherichia coli* (ETEC) through increasing expression of the tight junction protein ZO-1 and heat shock protein HSP27 (Liu et al., 2015). In an *in vitro* infection model, *L. reuteri* I5007 protected the mucosal barrier function against lipopolysaccharide (LPS) via maintenance of the expression of TNF- α and IL-6 and TJ proteins (Yang et al., 2015b).

A study on children with Crohn's disease has demonstrated that *L. reuteri* strain ATCC PTA 6475 can suppress TNF production by lipopolysaccharide-activated monocytes and primary monocyte-derived macrophages and that this is achieved by blockage of activation of MAP kinase-regulated c-Jun and the transcription factor and AP-1 (Lin et al., 2008a).

Clinical studies

Several human clinical trials have demonstrated the beneficial effects of *L. reuteri*, especially in infants and children. *Lactobacillus reuteri* has been proven to be an efficient probiotic. In treatment of infantile colic, ingestion of *L. reuteri* strains DSM 17938 and ATCC 55730 has been proven to be better than a placebo in reducing crying (Savino et al., 2010, Savino et al., 2007, Indrio et al., 2014). Oral administration of *L. reuteri* DSM 17938 appears to benefit gastrointestinal motility in infants, resulting in less overall regurgitation (37%) and improving bowel frequency (16%) (Indrio et al., 2014).

In a preliminary case-control study, treatment of infants with *L. reuteri* DSM 17938 decreased colonisation by EPEC (Savino et al., 2015). *Lactobacillus reuteri* DSM 17938 has also been shown to effectively reduce the duration of acute diarrhoea in children (Francavilla et al., 2012, Dinleyici and Vandenplas, 2014).

In a study on necrotising enterocolitis in newborn infants, *Lactobacillus reuteri* strains ATCC PTA 4659 and DSM17938 increased the survival rate and decreased the incidence and severity of the disease (Liu et al., 2012).

The only published study on effects on IBD shows that rectal infusion of *L. reuteri* in children with active distal ulcerative colitis enhances the anti-inflammatory cytokine IL-10, downregulates the pro-inflammatory cytokines IL-1 β , TNF α and IL-8, and improves mucosal inflammation (Oliva et al., 2012).

2 Aims

In recent years, research on the benefits of probiotics has grown rapidly and, as a result, the use of probiotics is increasing among the public. In order to confer their beneficial effect, probiotic bacteria needs to interact with the host cells. This so-called probiotic-host cross-talk is complex and knowledge about the details is limited. The overall aim of this thesis work was to provide new information on probiotics-host interactions and, in particular, mechanisms of *L. reuteri* probiosis in the GI tract.

As a part of this research, *L. reuteri* strains were equipped with reporter genes and genes encoding putative probiosis factors were inactivated. These new variants of *L. reuteri* were used to investigate the transit, location and interactions with the host GI tract. Furthermore, the effects of *L. reuteri* strains on complex junction proteins and immune mediators, and the importance of these interactions on mucosal permeability, were investigated. Specific objectives of the studies described in Papers I, II, III and the Preliminary study were to:

- Develop new genetic tools through *e.g.* generation of genetically modified *L. reuteri*, including strains expressing luminescence and fluorescence and strains in which genes of putative importance for probiosis were inactivated (Paper I and Paper III).
- Investigate these labelled *L. reuteri* strains by:
 - a) *In vivo* and *ex vivo* imaging, to study the real-time transit and location of *L. reuteri* along the GI tract (Paper I).
 - b) *In vitro* flow cytometry, to study plasmid persistence and fluorescence microscopy in order to evaluate adherence of the bacteria to epithelial cells (Paper I).
- Investigate the effect of *L. reuteri* strains on the mucosal barrier, apical junctions and immune mediators using an infection model (Paper II).
- Investigate the importance of specific genes, putatively involved in *L. reuteri* probiosis, for a protective effect on mucosal integrity and acute colitis (Paper III).
- Investigate the effect of *L. reuteri* strains on activation and degranulation of mast cells *in vitro* (Preliminary study).

3 Outline of materials and methods

In this chapter, the materials and methods used to conduct the work described this thesis, including in the preliminary study investigating the interactions between *L. reuteri* and mast cells, are reviewed. The methods are described in detail in Papers I, II and III.

3.1 Bacterial growth conditions (Papers I and II, Preliminary study)

All *Escherichia coli* strains were cultured on Luria-Bertani agar or in broth with shaking at 37 °C. All *L. reuteri* strains and mutants were cultured at 37 °C in de Man Rogosa Sharpe (MRS) broth or under anaerobic conditions on MRS agar. Strains harbouring plasmids were grown in the presence of erythromycin (400 µg mL⁻¹ for *E. coli* and 10 µg mL⁻¹ for *L. reuteri*).

3.2 Genetic engineering of *L. reuteri*

3.2.1 Construction of plasmids and generation of *L. reuteri* fluorescent and luminescent strains

A CBRluc::mCherry cassette was synthesised and cloned into the inducible vector pSIP411. The construct was first electroporated into two intermediate cloning hosts: *E. coli* PK401 and *Lactococcus lactis* MG1363. After creating also constructs with separated CBRluc and mCherry markers, all three variants were then electroporated into the *L. reuteri* strains R2LC and ATCC PTA 6475.

The recombinant strains were named 6475-CBRluc-mCherry, 6475-mCherry, R2LC-mCherry and R2LC-CBRluc. The sequences of the constructs were verified by PCR and sequencing.

3.2.2 Generation of mutant (Paper III)

Generation of targeted point mutations in the chromosome, without the need for selection, can be achieved by oligonucleotide-mediated recombineering (ssDNA recombineering) technology (van Pijkeren and Britton, 2012). The technique has been utilised for inactivation of a number of *L. reuteri* 6475 genes, *e.g.* in other studies cell- and mucus-binding protein A (*cmbA*; hmpref0563_10633), LPxTG-anchored amidase (hmpref0536_10802), propanediol dehydratase, large subunit (*pduC*) (involved in production of reuterin) and sortase (*srtA*); and in this study chaperon DnaK (*dnaK*).

3.3 Bacteria preparations for IPEC-J2, mast cell and murine models

The strains of *L. reuteri* were grown in 10 mL MRS broth, the optical density (OD) was measured and the bacterial cells were washed once with PBS. Prior to the experiment, the IPEC-J2 monolayer (Papers I-III) and mast cells (Preliminary study) were washed and cultured with antibiotic-free medium overnight.

The mast cells were pre-treated with *L. reuteri* ATCC PTA 6475, DSM 17938, 1563F and R2LC for 6 hours at a multiplicity of infection (MOI) of 50 (Preliminary study) and the epithelial cells were pre-treated with *L. reuteri* strains ATCC PTA 6475, DSM 17938 and 1563F (Paper II), and the 6475 mutants (6475 *dnaK*⁻, 6475 *pduC*⁻, 6475 amidase⁻, 6475 *cmbA*⁻ and 6475 *srtA*⁻) (Paper III) with 100 MOI.

For Paper II and Paper III, an infection model was established by infecting the IPEC-J2 cells with ETEC 853/67, a clinical isolate (Handl et al., 1988), at MOI 10 for 4 or 6 hours.

In Paper II, in order to measure the expression of tight junction (TJ) proteins and inflammatory cytokines, IPEC-J2 cells were pre-treated with *L. reuteri* strains for 6 hours at 100 MOI. Cells were thereafter washed twice with antibiotic-free medium and challenged with ETEC (10 MOI) for 4 hours.

In Paper III, the *L. reuteri* strains were cultured in 400 mL MRS broth overnight at 37 °C. The bacteria were then washed with PBS and suspended in 4 mL freezing buffer with glycerol and stored frozen (-70 °C) until use.

3.4 Animal experiments (Papers I and III, Preliminary study)

3.4.1 Animals

C57BL/6 female mice weighing between 25 and 35 g (Preliminary study), BALB/c male mice weighing between 18 and 35 g (Paper I) and 60 C57BL/6 male mice aged 6 to 8 weeks (Paper III) were used. The animals were maintained under standardised conditions at 21-22 °C with daily illumination of 12 h darkness/12 h light. The animal experiments were performed with the permission of the Swedish Laboratory Animal Ethics Committee in Uppsala and according to the guidelines of the Swedish National Board for Laboratory Animals.

3.4.2 *In vivo* and *ex vivo* imaging of *L. reuteri* strains in mice (Paper I)

The 6475-CBRluc-mCherry and R2LC-mCherry strains were diluted in PBS and the induction peptide and D-Luciferin were added to 1 mL of bacterial suspension. Different doses of recombinant *L. reuteri* (1×10^5 - 1×10^{10} CFU) were used. The bacterial mixtures were gavaged orally following anaesthesia of the mice with 2% isoflurane. In order to study the transit of the recombinant bacteria in the GI tract, *in vivo* images were taken 0, 60, 120 and 180 minutes post-gavage by a Spectrum pre-clinical *in vivo* imaging system (IVIS). For *ex vivo* imaging, the GI tract was removed at different time points (0, 60, 120 and 180 min post-gavage) and images were taken using an IVIS camera, with air injection or not, as described in earlier studies (Foucault et al., 2010, Rhee et al., 2011). The *in vivo* fluorescence and luminescence were expressed as average radiance (p/sec/cm²/sr).

3.4.3 DSS-induced colitis and evaluation of the disease activity index (DAI) (Paper III)

Three independent experiments were performed using in total 60 mice randomly distributed into different treatment groups. The experimental time line is described in Figure 4. In brief, three days prior to induction of colitis the mice were gavaged with PBS (control group), *L. reuteri* 6475 wild type and the three mutants 6475 *cmbA*⁻, 6475 *dnaK*⁻ and 6475 *pduC*⁻, all with a dose of 1×10^8 CFU. The acute colitis was induced by adding 2.5-3% (w/v) DSS to the drinking water for 7 days.

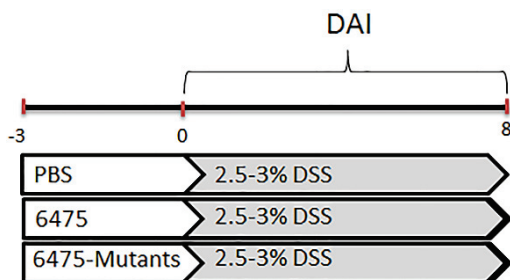


Figure 4. Time plan of the colitis experiment. Colitis in mice was induced by dextran sulphate sodium (DSS) after pre-treatment with *Lactobacillus reuteri*.

The severity of the colitis was examined by evaluating the disease activity index (DAI) each day during a period of 8 days with a scale (0-4) described previously by Cooper and co-workers (Cooper et al., 1993, Cooper et al., 2000).

3.5 Mammalian cell culture

3.5.1 Epithelial cell culture (Papers I- III)

The IPEC-J2 cell line was employed for evaluation of the interaction with epithelial cells. This is a nontransformed epithelial cell line that has been derived from the small intestine of a unsuckled piglet (Berschneider, 1989). IPEC-J2 cells are analogous to the human gut physiology (Zakrzewski et al., 2013). The cells were cultured and maintained in complete Dulbecco's modified Eagle's medium (DMEM)/F-12 Ham at 37 °C in an atmosphere of 5% CO₂.

5×10⁵ cells were seeded per transwell filter and thereafter allowed to polarise (Papers II and III). A high seeding density is required to prevent cell proliferation (Cerejido et al., 1978). It has been shown that IPEC-J2 cells grown on transwell filters can differentiate to enterocytes that exhibit enterocyte characteristic such as microvilli, TJ and production of cytokines (Roselli et al., 2007, Brosnahan and Brown, 2012).

3.5.2 Isolation and generation of murine peritoneum-derived mast cells (PCMCs) (Preliminary study)

In the Preliminary study, a primary murine mast cell model (peritoneal cell-derived mast cells) (PCMCs) was employed to study the effect of *L. reuteri* on mast cell activation and degranulation. Resident peritoneal mast cells are mature serosal-type and differentiated mast cells that can be expanded in culture to

generate homologous cells and the characteristics of peritoneal mast cells is almost retained (Malbec et al., 2007). Peritoneal cells were collected from wild-type C57BL/6 mice and PCMCs were isolated and established according to a protocol described elsewhere (Malbec et al., 2007). In brief, PCMCs were established by culture of peritoneal cells in DMEM plus GlutaMAX (Gibco, Invitrogen) supplemented with 10% supernatant of transfected Chinese hamster ovary cells (CHO cells) with an expression vector constitutively expressing murine stem cell factor (SCF) (kindly provided by Dr. M. Daeron, Pasteur Institute, France), 60 µg/mL streptomycin, 50 µg/mL penicillin, 10% FBS, 100 µM MEM non-essential amino acids and 50 µM 2-mercaptoethanol. A culture time of 4-5 weeks was used for the experiment and the medium was changed every 4-5 days.

3.6 *In vitro* exposure of PCMCs to *Lactobacillus reuteri* and measuring of activation and degranulation

3.6.1 Activation of mast cells (preliminary study)

For the Preliminary study, PCMCs were washed three times in PBS and centrifuged for 8 min at 0.4 rcf. Mast cells were re-suspended in antibiotic-free medium at a density of 1.5×10^6 cells/mL and sensitised with IgE anti-DNP (BD Biosciences, Pharmingen) at a concentration of 1 µg/mL overnight at 37 °C. Next day, 0.75×10^5 CFU/mL of *L. reuteri* strains (ATCC PTA 6475, DSM 17938, R2LC and 1563F) corresponding to 50 MOI were added to the mast cells for 6 hours (based on our primary results). After 6 hours of incubation, samples were collected for the viability test on mast cells using trypan blue staining. The excess of IgE, anti-DNP and bacteria was washed away using Tyrode's buffer three times. The mast cells were re-suspended in Tyrode's buffer and plated at a density of 2×10^6 cells/mL into 24-well tissue plates in triplicate for each treatment. To stimulate the mast cells, 0.5 µg/mL DNP-HAS was added to the cells, followed by a 30 min incubation at 37 °C. For calcium ionophore-mediated degranulation, Ca^{2+} ionophore (A23187, Sigma Aldrich) was added (2 µM final concentration) to the cells. After stimulation, cells collected by centrifugation and both supernatant and cell pellets were saved at -20 °C until analysed.

For the qPCR experiments, mast cells were stimulated or not stimulated as described above, followed by 2 hours of incubation. Cells were collected by centrifugation, frozen and stored at -70 °C.

3.6.2 β -hexosaminidase assay (Preliminary study)

In the Preliminary study, a β -hexosaminidase assay was performed. The PCMC pellets were lysed in 250 μ L of 1% Triton X-100 in Tyrode's buffer. Then 20 μ L samples of cell lysate (four-fold dilutions) or supernatant were loaded in duplicate into a 96-well plate (flat bottom, Nunc), followed by 80 μ L of the substrate 1 mM p-nitrophenyl N-acetyl- β -D-glucosamine (Sigma Aldrich) diluted in 0.05 M citrate buffer (pH 4.5), and the plate was incubated at 37 °C for 1 hour. The reactions were stopped and quenched with 200 μ L of 0.05 M NaCO₃ buffer (pH 10) and the plate was read at 405 nm. Results are shown as the percentage of β -hexosaminidase released from the cells.

3.7 Expression of complex junction proteins and pro-inflammatory cytokines

3.7.1 Extraction of protein and immunoblotting

In Paper II, total protein in IPEC-J2 cells was extracted using lysis buffer from the mirVana PARIS Kit. The cell lysate was centrifuged and the supernatant collected. The protein concentration was measured with a protein assay kit using a Qubit 3.0 Fluorometer. The proteins were separated by electrophoresis and transferred to PVDF membranes.

After blocking, the membranes were incubated with the desired antibodies. The primary antibodies used in Paper II were as follows: rabbit polyclonal anti-ZO-1, anti-cadherin-1 and anti- β -actin. The loading control was β -actin. Thereafter, the membranes were washed and incubated with HRP-conjugated goat anti-rabbit sera and washed. A chemiluminescence detection reagent (ECL) was used to visualise protein bands. Densitometry was carried out to quantify the bands.

3.7.2 RNA isolation and expression analysis by real-time PCR

The total RNA from the IPEC-J2 (Paper II) and mast cells (Preliminary study) was isolated using a NucleoSpin RNA II Kit according to the manufacturer's instructions. Approximately 1 μ g RNA was reverse-transcribed to cDNA. In the Preliminary study, the following PCR protocol was used: 95°C, 10 min; 40 times (95°C, 30 s; 57°C, 20 s; 72°C, 20 s). For both studies, a melting curve programme (60-95°C with a heating degree of 0.1°C/s and continuous fluorescence measurement) was used. Relative gene expression was quantified using three technical replicates for each sample. The relative expression ratio of

IL-6 and TNF α (Paper II), IL-6 and IL-13 (Preliminary study) was calculated based on the Ct deviation of treated samples compared with untreated and real-time PCR efficiencies and normalised against the expression levels of the reference genes β -actin (Paper II) and GAPDH (Preliminary study) using methods $2^{-\Delta\Delta C_T}$ (Preliminary study) and Pfaffl (Paper II).

Table 1. List of primers used in the Preliminary study on mast cell-Lactobacillus reuteri interactions

Gene name	Source	Primer sequence	Product size (bp)	TM ($^{\circ}$ C)
IL-6	Mouse	F:5'AGACAAAGCCAGAGTCCTTCAGAGA-3	148	71
		R:5' TAGCCACTCCTTCTGTGACTCCAGC-3'		
IL-13	Mouse	F:5' CAGCATGGTATGGAGTGTGG-3'	117	67
		R:5' AGGCCATGCAATATCCTCTG-3'		
GAPDH	Mouse	F:5' CACTGAGCATCTCCCTCACA-3	111	68
		R:5' GTGGGTGCAGCGAACTTTAT-3'		

3.8 Statistical analyses

All experiments in Paper II were performed with triplicate or quadruple independent seedings with three technical replicates. In Paper III, the comparison of 6475 wild types and the mutants was performed with three technical replicates. Statistical differences in transepithelial electrical resistance (TEER) and FITC-dextran between all treatments were assessed using one-way ANOVA followed by a Student's *t*-test (Papers II and III). Statistical analyses of the real-time qPCR (Paper II and Preliminary study) and immunoblotting experiments (Paper II) were conducted with one-way ANOVA analysis followed by Tukey's multiple comparisons post-test and a Student's *t*-test, respectively. Significant differences between groups analysed for effects on DAI (Paper III) and mast cell degranulation (Preliminary study) were evaluated using two-way ANOVA and one-way ANOVA, followed by Tukey's multiple comparisons post-hoc test respectively. In all experiments, differences were considered significant at $p \leq 0.05$ and data were presented as mean \pm standard error of the mean (SEM). All statistical analyses were carried out with the JMP statistical software Pro 11.

4 Main Results

This chapter provides an overview of the main results of Papers I-III and the Preliminary study.

4.1 *In vitro* and *in vivo* application of fluorescent and luminescent variants (Paper I)

4.1.1 *In vitro* applications of fluorescence- and luminescence-labelled *L. reuteri* and assessment of plasmid persistence

The signal intensity of the recombinant luminescent and fluorescent *L. reuteri* generated and the persistence of the pSIP-CBRluc-mCherry, pSIP-CBRluc and pSIP-mCherry plasmids were evaluated in the absence of selection pressure. The 6475-CBRluc-mCherry, 6475-CBRluc and R2LC-mCherry strains were cultivated for 10 days in the absence of antibiotics, which caused loss of the plasmids in a time-dependent manner, as shown by flow cytometry (FCM), plate counts (PC) and a luciferase assay. Data from the luciferase assay showed that the smaller plasmid, pSIP-CBRluc, was more stable in R2LC-CBRluc than the larger plasmid, pSIP-CBRluc-mCherry, in 6475-CBRluc-mCherry after bacterial serial subculture (77% compared with 12% at day 1, 7% compared with 0% at day 10).

The two methods PC and FCM were compared in experiments evaluating plasmid persistence in fluorescence-producing bacteria. The persistence of pSIP-mCherry in R2LC-mCherry and 6475-mCherry was detected to be ~20 and ~35 %, respectively, when using PC and 60 and 95 %, respectively, when using FCM, after 4 days of bacterial subculture. For 6475-CBRluc-mCherry, the plasmid could not be detected at day 4 of serial subculture, as shown by both PC and FCM assays. In contrast, over a period of 10 days of daily subculture of the bacteria in the presence of antibiotics, signal intensity was stable for all

fluorescent recombinants and 6475-CBRluc-mCherry exhibited the lowest (1.8×10^3 p/s) and R2LC-mCherry the highest (2.9×10^4 p/s) signal intensity ($p < 0.001$) shown by FCM. The signal stability for the fluorescent bacteria was evaluated by fluorescence microscopy (FLM) and signal heterogeneity was observed among plasmid-bearing cells.

Furthermore, the mCherry signal was affected by both pH and duration of induction time. An improvement in signals was observed at neutral pH compared with pH 4.6 when using FLM. In addition, the signal intensity improved significantly ($p < 0.001$) with a long induction period (~20 hours) compared with a short induction period (~4 hours) for the mCherry-producing strain (6475-mCherry).

4.1.2 *In vivo* applications of recombinant *L. reuteri*

In vivo and *ex vivo* transit and location of luminescence-producing strains of *L. reuteri* were assessed and visualised after intragastric administration of different doses of the bacteria (from 1×10^5 to 1×10^{10} CFU/mouse) using an *in vivo* imaging system (IVIS). Using whole body imaging, the highest luminescence signal from 6475-CBRluc-mCherry and R2LC-CBRluc (3.8×10^8 and 7.6×10^6 p/sec/cm²/sr, respectively) and the fluorescence signal from 6475-CBRluc-mCherry (2.6×10^8 p/sec/cm²/sr) were detected in the high dose treatment (1×10^{10} CFU/mouse). Interestingly, the luminescence signal of the lowest dose of 6475-CBRluc-mCherry (1×10^5 CFU/mouse) could also be detected.

Observations of the transit and location of the two recombinant strains 6475-CBRluc-mCherry and R2LC-CBRluc varied and indicated a faster transit time for 6475-CBRluc-mCherry compared with R2LC-CBRluc at 60 min post-gavage. Moreover, at 3 h post-gavage, the bioluminescence signals of mice given the high dose could still be visualised *ex vivo* and large numbers of bacteria were retained in the stomach.

The fluorescence signal from 6475-CBRluc-mCherry was not detectable after 60 min.

4.2 *Lactobacillus reuteri* and intestinal permeability

4.2.1 Protective effect of *L. reuteri* on epithelial permeability

An infection model of polarised IPEC-J2 monolayers using the ETEC strain 853/67, a porcine clinical isolate capable of producing different types of enterotoxins (Handl et al., 1988), was used. Two different doses (100:1 and

1000:1 MOI) of the *L. reuteri* strains ATCC PTA 6475, DSM 17938, R2LC and 1563F were employed for pre-treatment of the cells.

The results indicated that two hours post ETEC-challenge, TEER was reduced by 5% for the *L. reuteri* groups and by 24% in the ETEC-group. After 4 hours, TEER declined further and reached 96% in the ETEC-group and the pre-treatment with all *L. reuteri* strains could only partly protect the epithelial monolayer. A high dose of bacteria preserved higher TEER values compared with lower dose significantly ($p < 0.05$).

The protective effect of the *L. reuteri* strains against the deleterious effect of ETEC on monolayer integrity was more pronounced when measuring the tracer FITC-dextran. After 6 hours post-infection, the FITC-dextran permeability was decreased by 60-85% for the high dose and 50-70% for the low dose compared with the ETEC group. Among the *L. reuteri* strains tested, ATCC PTA 6475 and DSM 17938 showed the highest protective efficacy (Paper II).

In Paper III, a preliminary experiment was carried out to compare the protective effect of *L. reuteri* 6475 wild type with its derivative mutants on the detrimental effect of ETEC on mucosal integrity. In the five mutants evaluated, the genes encoding the following proteins had been inactivated: chaperone DnaK (*dnaK*⁻), propanediol dehydratase, large subunit (*pduC*⁻; involved in production of reuterin) cell- and mucus-binding protein A (*cmbA*⁻), LPxTG-anchored amidase (*hmpref0536_10802*⁻) and sortase (*srtA*⁻).

Two and four hours post-ETEC challenge of monolayers, there was a decrease in TEER of ~60% and ~96 %, respectively, whereas pre-treatment of the monolayers with *L. reuteri* wild type and mutants followed by ETEC challenge resulted in a decrease of 10-28% and ~94%, respectively. However, there were no significant differences between the mutants and the wild type. In addition, FITC-dextran permeability was significantly higher for the ETEC control group (100%) compared with the *L. reuteri* group (14-26%; $p < 0.05$). No significant difference was found between treatments with wild type and the mutants (Paper III).

4.2.2 *Lactobacillus reuteri* reverses the deleterious effect of ETEC on expression of apical junction proteins

Expression of E-cadherin and zonula occludens (ZO-1) was quantified by Western blot tests (Paper II). Densitometry analysis of the immunoblots revealed two bands of size 195 and 187 kDa, corresponding to two different isoforms of ZO-1. First, destruction of monolayer integrity was confirmed by evaluating the expression of E-cadherin and ZO-1 following ETEC infection. Overall, the expression of both E-cadherin and ZO1-187 declined by more than 60%. This

effect was reversed by pre-treatment of the monolayers with *L. reuteri* strains DSM 17938 and 1563F, giving two- and three-fold higher expression of E-cadherin and ZO1-195, respectively ($p < 0.05$).

In addition, cells pre-treated with strain 1563F exhibited a three-fold increase in expression of ZO1-187 compared with the ETEC control group ($p < 0.05$).

In Paper II, in order to investigate whether *L. reuteri* could prevent an inflammatory response in the epithelial monolayer, IPEC-J2 cells were pre-treated with *L. reuteri* for 6 h, followed by infection with ETEC for 4 h, after which the mRNA expression of the pro-inflammatory cytokines TNF α and IL-6 was evaluated by real-time PCR. Infection of the cells with ETEC increased TNF α and IL-6 expression 21-fold and ~six-fold, respectively, compared with the control ($p < 0.05$).

Pre-treatment of the cells with strain DSM 17938 followed by ETEC challenge gave a two-fold reduction in IL-6 gene expression ($p < 0.05$). In addition, pre-treatment with all three *L. reuteri* strains decreased mRNA expression of TNF α two- to three-fold compared with the ETEC group ($p < 0.05$). *Lactobacillus reuteri* downregulates elevated expression of pro-inflammatory cytokines induced by ETEC.

4.2.3 Mode of action of *L. reuteri* in a DSS-induced acute colitis murine model (Paper III)

A ssDNA recombineering technique was used to introduce a site-directed mutation in the *dnaK* gene encoding the heat shock protein DnaK. *Lactobacillus reuteri* 6475 wild type and its derivative mutants of chaperon DnaK (*dnaK*⁻); propanediol dehydratase, large subunit (*pduC*⁻); and cell- and mucus-binding protein A (*cmbA*⁻) were selected for *in vivo* studies of the probiosis mechanism involved in amelioration of inflammation and tissue damage in colitis (Paper III). A model of acute colitis was established in mice by administration of 2.5-3% DSS in drinking water for 8 days. The disease activity index (DAI) was calculated by scoring changes in weight loss, stool consistency and blood content, as described elsewhere (Cooper et al., 1993, Cooper et al., 2000).

Three days of pre-treatment of the animals with *L. reuteri* 6475 (prior to induction of acute colitis by DSS) significantly lowered DAI score at day 8 compared with DSS group. In addition, no significant differences between wild type 6475 and the three mutants were observed. However, two of the mutants, 6475 *cmbA*⁻ and 6475 *dnaK*⁻, had a tendency to decrease their protective effects and increase the DAI scores.

4.3 *Lactobacillus reuteri* and mast cell activation and degranulation

The aim of the Preliminary study was to determine whether *L. reuteri* inhibits mast cell degranulation as well as pro-inflammatory cytokine synthesis and release.

The majority of mast cells are present in mucosal layers, where the body encounters the outside environment. Mast cells are a granulated cell type of the immune system that can release a variety of pre-made and *de novo* synthesised mediators such as histamine, β -hexosaminidase, proteases, cytokines and chemokines upon activation based on the type of stimuli (*e.g.* IgE-mediated or Toll-like receptor-mediated) (Wernersson and Pejler, 2014).

A pilot study was performed on murine peritoneal cell-derived mast cells (PCMC) with three technical replicates. The interaction between PCMCs and *L. reuteri* was assessed and production of some of the mast cell mediators including β -hexosaminidase and pro-inflammatory cytokines IL-6 and IL-13 was studied. To examine the effect of *L. reuteri* on mast cell degranulation, cells were pre-treated with *L. reuteri* prior to the IgE-mediated stimulation and β -hexosaminidase release from the cells was measured. As a positive control, Ca²⁺ ionophore was used, which caused strong degranulation.

Six hours of pre-treatment of the mast cells with four strains of *L. reuteri* (ATCC-PTA 6475, DSM17938, R2LC and 1563F) did not affect the viability of mast cells (Figure 5A). *Lactobacillus reuteri* strains did not degranulate the mast cells prior to stimulation except for 6475, which caused 20% β -hexosaminidase release. Moreover, *L. reuteri* strains did not prevent the degranulation of PCMCs after mast cells had been stimulated (Figure 5B).

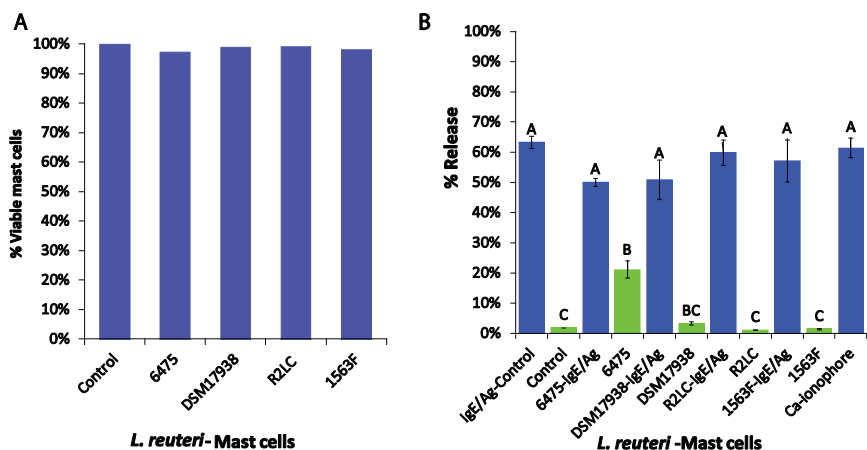


Figure 5. Effect of *Lactobacillus reuteri* on mast cell viability and degranulation. The peritoneal cell-derived mast cell (PCMCs) were pre-treated with four different strains of *L. reuteri* and then stimulated using IgE anti-DNP followed by DNP-HAS (IgE/Ag). Prior to stimulation, the viability of mast cell was evaluated (A). The β -hexosaminidase activity of the PCMCs was assessed before and after stimulation (B). Data are represented as relative activity in supernatant/total activity and represented as the mean (\pm SEM) of three technical replicates. Columns that do not share similar letters are significantly different ($p \leq 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test.

Pre-treatment of mast cells with *Lactobacillus reuteri* downregulated the levels of both IL-6 and IL-13. More specifically, a trend of lower expression levels of both IL-6 and IL-13 was seen for cells treated with all of the *L. reuteri* strains compared with the untreated cells. However, although the pre-treatment lowered the amount of target RNA compared with the untreated cells, no significant differences were found between untreated cells and cells pre-treated with *L. reuteri* except for expression of IL-6 from cells pre-treated with R2LC ($p < 0.05$) (Figure 6A and 6B).

Comparing the stimulated cells (both pre-treated and untreated) to the control revealed upregulation of IL-6 and IL-13 by approximately 10-fold in IgE/Ag-control compared with the control. Interestingly, two of the *L. reuteri* strains were able to reduce this prominent increase in both cytokines after stimulation compared with the IgE/Ag-control. *Lactobacillus reuteri* 6475 counteracted the upregulation of IL-13 and IL-6 caused by stimulation, to levels lower than that of the control (17-fold and 20-fold, respectively) (Figure 6A and 6B). The 1563F strain also downregulated expression of IL-13 and IL-6 by \sim three-folds. In addition, a trend for decreased expression was observed for DSM17938 and R2LC (Figure 6A and 6B).

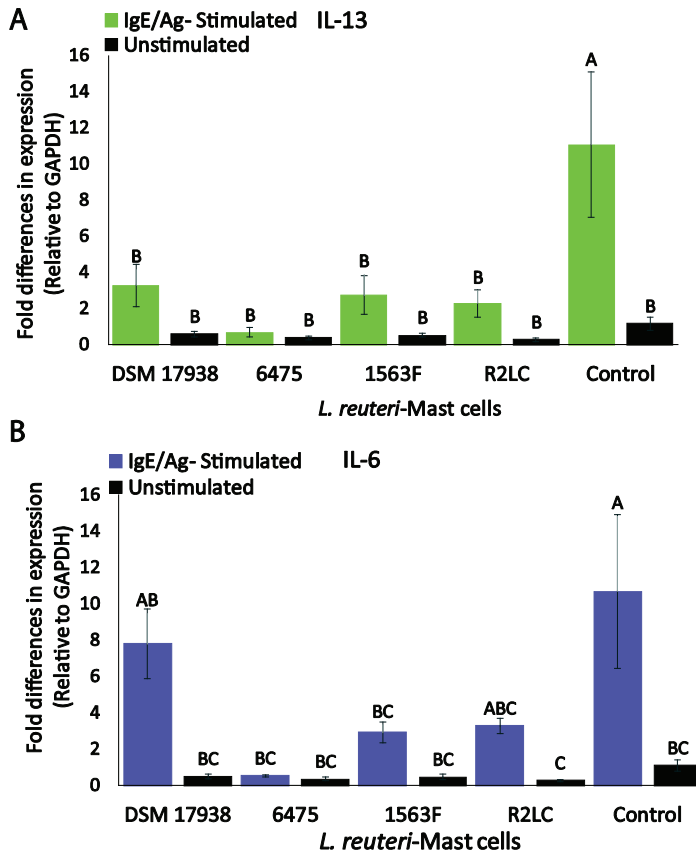


Figure 6. Effect of *Lactobacillus reuteri* on gene expression of peritoneal cell-derived mast cell (PCMC) pro-inflammatory cytokines. The PCMC treated or not with *L. reuteri* strains for 6 h. The expression of IL-13 (A) and IL-6 (B) in response to IgE anti-DNP and DNP-HAS (IgE/Ag) stimulation or in the absence of stimuli was evaluated against an IgE/Ag control. The results were considered significant at $p < 0.05$ by one-way ANOVA with Tukey's post-hoc test and columns labelled with different letters are significantly different. The data represent the mean (\pm SEM) of three technical replicates.

5 Main Discussion

5.1 Genetic engineering of *L. reuteri* and *in vivo* and *in vitro* applications of recombinant *L. reuteri* (Paper I)

In Paper I, *in vivo* and *in vitro* applications of recombinant fluorescent and luminescent *L. reuteri* were employed and the recombinant bacteria were used to study plasmid persistence and transit and location of *L. reuteri* in the GI tract of mice.

The work in Paper I started with genetic engineering of *L. reuteri* and labelling of *L. reuteri* with reporter genes, namely the bioluminescence reporter click beetle luciferase (CBRed) and red fluorescent mCherry, which have recently been used for experimental bioluminescent and fluorescent imaging in the probiotics field.

A large number of reporter proteins (*e.g.* luminescence and fluorescence proteins) are available as markers that allow easy detection of labelled cells in complex microbial ecosystems. From the first discovery of green fluorescent protein (GFP) (Shimomura et al., 1962), a range of fluorescent proteins have been discovered and developed and have been expressed in prokaryotic and eukaryotic organisms (Chalfie, 1994). However, most research on fluorescence reporter genes has been directed toward optimisation of fluorescence, improvement of photo-stability, decreasing the maturation time and increasing the brightness (Shaner et al., 2005). mCherry was chosen in this thesis for numerous reasons. It has a monomeric structure that make it an ideal choice for protein fusions, it is non-toxic to its host even in high amounts, it is resistant to photo bleaching, it has high stability and it is easy to detect under diverse *in vivo* and *in vitro* conditions (Shaner et al., 2005).

Besides mCherry, the CBRed from *Pyrophorus plagiophthalmus* was chosen. The reason for the choice was the versatile use of this protein in gene expression studies and for bioluminescence imaging (BLI), despite the

inevitable need for oxygen for the reaction between the luciferase enzyme and its substrate to generate visible light (Gheysens and Mottaghy, 2009).

In Paper I, the mCherry and CBRluc genes were codon-optimised for *Lactobacillus reuteri* and successfully expressed in the R2LC and 6475 strains. The importance of codon optimisation has been shown previously and fluorescence and luminescence genes have been codon-optimised for a number of bacteria, including lactobacilli, in order to maximise expression of the reporter (Landete et al., 2016). A good example is codon optimisation in *Mycobacterium tuberculosis*, which increases expression of a luminescence gene by 30-fold (Andreu et al., 2010).

After generation of the fluorescence-labelled *L. reuteri*, its possible use in evaluation of plasmid persistence was investigated. For this, an alternative technique based on flow cytometry (FCM) for assessing plasmid persistence was introduced and compared with the classical plate count (PC) method and replica plating. The hypothesis tested was that FCM is a good alternative to the PC method and that the latter laborious method can be replaced by FCM for large-scale screenings in some situations. It was found that the plasmid stability profiles for all recombinant strains were virtually identical (when evaluated by both FCM and PC), showing that the plasmids were gradually lost and that a selection force is required for plasmid persistence. When the two methods were compared, the only difference found was a higher plasmid loss rate of 6475-mCherry detected by PC compared with FCM. A previous study also found similar plasmid persistence profiles by both PC and FCM (Loftie-Eaton et al., 2014).

Plasmid persistence in bacterial populations has previously been studied using replica plating (Cooper and Heinemann, 2000, Deane and Rawlings, 2004). In agreement with previous research, Paper I showed that the conventional culture-based methods have several major drawbacks, such as being time-consuming, labour-intensive and challenging to use for high-throughput screening. Furthermore, the PC method usually generates uncertain results associated with differences between low-plasmid/free-plasmid and high-plasmid containing bacterial growth rates, where the latter gradually accumulates in the culture (Lau et al., 2013a). Thus, other methods such as FCM, culture-based microscopy and real-time quantitative PCR (qPCR) have recently been tested and suggested to be possible alternatives for assessing plasmid persistence (Bonot and Merlin, 2010, Loftie-Eaton et al., 2014, Bahl et al., 2004, Lau et al., 2013b).

It was concluded in Paper I that FCM is a competitive alternative to plate count assays for studies of plasmid persistence. Some of the advantages of using FCM in studying recombinant labelled *L. reuteri* and plasmid persistence are:

the high sensitivity, allowing detection of bacteria with low fluorescence signals in complex bacterial populations, allowing large-scale screening, and the absence of a need for selectable markers, making it a powerful analytical technique for evaluation of plasmid persistence.

Despite the presence of many reporter proteins, application of bioluminescence reporters for *Lactobacillus* species is essentially undeveloped and Paper I describes the only successful luminescence construct for *L. reuteri*. Besides that, there are only a few reports for other *Lactobacillus* species (Daniel et al., 2013, Landete et al., 2015). However, there are more reports of utilising different fluorescence-producing bacteria *in vivo*. Green fluorescent protein has been used to detect bacteria in chickens (Yu et al., 2007) and goats (Han et al., 2015). Plasmids containing mCherry have also been used to study the colonisation of *L. plantarum* in a germ-free zebrafish model (Russo et al., 2015) and in the GI tract of mice (van Zyl et al., 2015).

Next, the successfully generated *L. reuteri* 6475-mCherry, 6475-CBRluc-mCherry, R2LC mCherry and R2LC-CBRluc were used for *in vivo* and *ex vivo* studies. It was found that expression of the reporter proteins from these recombinant strains facilitated tracking of the bacteria in the GI tract. This is an important approach to study interactions between probiotic species and the host.

The Caliper *in vivo* imaging system (IVIS), which is one of the most widely used optical imaging systems, is equipped with a highly sensitive thermoelectrically cooled CCD camera (Caliper Life Sciences, Hopkinton, MA, USA) (Gheysens and Mottaghy, 2009). The IVIS approach allowed monitoring, in real time, of the transit of different doses of both luminescent and fluorescent *L. reuteri* and a dose as low as 10^5 luminescent bacteria/mouse or a dose of 10^{10} fluorescent bacteria/mouse could be detected.

5.2 *Lactobacillus reuteri* counteracts mucosal inflammation induced by an enteric pathogen (Paper II)

The intestinal barrier consist of several layers and is highly regulated (Scaldaferri et al., 2012). It is known that a balance between selective passage of immunogens across the mucosal barrier and the resulting immune response is necessary for intestinal haemostasis (Menard et al., 2010). A non-selective increase in passage of antigens, as well as microbes, causes inflammation and further disruption of the mucosa.

Paper II studied mucosal integrity and function in response to enterotoxigenic *E. coli* (ETEC) and *L. reuteri* *in vitro* and investigated whether *L. reuteri* could

ameliorate the detrimental effect of ETEC on the mucosal barrier in the course of an *in vitro* infection. ETEC 853/67 is a toxin-producing isolate (Handl et al., 1988), which was used in Paper II for establishment of an infection model of non-transformant small intestinal cells of pig origin (IPEC-J2) (Geens and Niewold, 2010, Brosnahan and Brown, 2012). The model has been used previously to study the effects of probiotics on the intestinal barrier (Liu et al., 2014, Yang et al., 2015b, Zhang et al., 2015).

Initially, the effect of the pathogen on the mucosal barrier was evaluated and it was observed that the destruction of the mucosal barrier by ETEC was linked to modulation of the paracellular and transcellular pathways. This was detected by a decrease in transcellular electric resistance (TEER) and increase in permeability to FITC-dextran (4 kDa), as found in previous studies (Liu et al., 2015, Yang et al., 2015a).

The findings in Paper II indicated that the damage to the mucosal barrier by ETEC was associated with modulation of apical junction complex proteins, which was detected as a strong decrease in E-cadherin and ZO1-187.

Previous studies have demonstrated that pathogens, including ETEC, can alter the intestinal barrier function through different mechanisms, for instance either through production of toxins that are able to activate the myosin light chain kinase, which leads to weakening of the endothelial cell-cell adhesion and barrier function (Johnson et al., 2010, Philpott et al., 1998, Shen et al., 2010) or production of proteases that alter the structure and function of junctional proteins and initiate re-organisation of the cytoskeleton (Blum and Schiffrin, 2003, de Vrese and Marteau, 2007). Furthermore, downregulation of mucin production, lysis of epithelial cells and induction of apoptosis, cell cycle arrest and growth inhibition, all of which facilitate invasion of pathogens into the intestinal cell layer, have been studied extensively (McGuckin et al., 2011).

In addition, hijacking of cellular molecules and signalling pathways of the host, including upregulation of pro-inflammatory cytokines, is often part of the pathogenic process (McGuckin et al., 2011). It was found in Paper II that disruption of the mucosal barrier by ETEC was associated with a strong increase in TNF α and IL-6 expression, and the afore-mentioned modulation of apical junction proteins, which is in agreement with previous studies (Shimazu et al., 2012, Wang et al., 2016).

How probiotics can communicate with epithelial cells and the mechanism by which *L. reuteri* exerts its probiosis activities on impaired epithelial barrier caused by infection were studied.

Paper II showed that *L. reuteri* could partly reverse or counteract the inflammatory and mucosal damage processes. Attenuation of inflammation of intestinal epithelial cells by *L. reuteri* was found to be through downregulation

of expression of TNF α and IL-6. Upon downregulation of the elevated expression of the pro-inflammatory cytokines by *L. reuteri*, the expression of ZO-1 and E-cadherin was stabilised, and as a result further damage to the intestinal barrier was slowed down. The results indicated that there might be a link between the immune mediators TNF α and IL-6 and the tight junction proteins (Paper II), a correlation reported previously in studies conducted *in vitro* (Suzuki et al., 2011, Tazuke et al., 2003, Ye and Ma, 2008) and *in vivo* (Wang et al., 2001, Yang et al., 2003).

In order to study the importance of the dose, the effects of a low (100 MOI) and a high dose (1000 MOI) of *L. reuteri* were compared. The higher dose showed better protection and maintenance of the monolayer in the late phase of infection. Of course, a study on cultivated epithelial cells is far from a human clinical trial, but investigations of the dose effect in simple models can provide valuable information. In fact, studies on human and animals have demonstrated that the beneficial effect of probiotics can be dose-dependent (Ouweland, 2016, Fang et al., 2009, Suo et al., 2012). Probiotics for humans have been used at doses between 10⁷ and 10¹¹ CFU (Weizman et al., 2005, Gionchetti et al., 2000, Ouweland, 2016).

As known, probiotics are described as living microorganisms which, when administered in adequate amounts, confer health benefits (Joint FAO/WHO Group, 2002). However, there is no description or definition of an ‘adequate dose’ for humans. Proper probiotic dosage with concern to safety and effectiveness has not been systematically studied in human, due to high complexity and the high costs of clinical trials. Apart from general criteria (body weight, age and health endpoint) possibly affecting the dose, genetic background, microbial community, diet, route of administration and, last but not least, the manufacturing process (*e.g.* fresh or freeze-dried bacteria) make the matter even more complex.

It was concluded in Paper II that *L. reuteri* exerts protective effect on leaky intestinal barrier induced by ETEC through stabilisation of tight junction ZO-1, adherens junction E-cadherin and the pro-inflammatory cytokines TNF α and IL-6.

5.3 Effect of *L. reuteri* wild type and mutants on mucosal integrity and inflammation using DSS-induced colitis and *in vitro* infection models (Paper III)

Paper III attempted to identify genes of *L. reuteri* 6475 involved in the beneficial effects seen on mucosal integrity and inflammation in Paper II and mucosal damage in an ulcerative colitis model. Therefore, five mutants in which the genes

encoding chaperone DnaK (*dnaK*⁻), propanediol dehydratase, large subunit (*pduC*⁻), cell- and mucus-binding protein (*cmbA*⁻), LPxTG-anchored amidase (amidase⁻), and sortase (*srtA*⁻) had been inactivated were evaluated in a mice colitis model and an infection model with IPEC-J2 cells.

The study was initiated by generation of mutants using a recombineering technology. A comparative pilot study of 6475 wild type and its derivative mutants, *dnaK*⁻, *pduC*⁻, *cmbA*⁻, amidase⁻ and *srtA*⁻ was carried out on polarised IPEC-J2 monolayers. It showed that pre-treatment of the monolayers with both the wild type and the mutants counteracted the inflammation and delayed the onset and progression of infection by ETEC.

In conclusion, no significant differences of FITC-dextran permeability, TEER and protection of monolayer integrity were observed between 6475 wild type and the mutants. This *in vitro* study was a pilot and should be repeated to confirm the results obtained.

In addition to the *in vitro* study, 6475 wild type and three of the mutants, *dnaK*⁻, *pduC*⁻ and *cmbA*⁻, were evaluated for their protective effect on DSS-induced acute colitis of mice.

During recent decades, several animal models of IBD have been developed and accepted. Chemically induced murine models of IBD are the most commonly used. In Paper III, chemically induced colitis was established by administration of 2.5-3% DSS, (C₆H₇Na₃O₁₄S₃)_n in the drinking water for 7 days. DSS is one of the most commonly used agents for the induction of acute and chronic IBD (Barnett and Fraser, 2011). It is believed that DSS acts through disruption of the epithelial integrity, and that the model clinically and histopathologically resembles ulcerative colitis (Neurath et al., 1995, Alex et al., 2009) and that the results obtained can be translated into human disease (Melgar et al., 2008).

A protective effect of *L. reuteri* 6475 wild type on DSS-induced colitis was observed in Paper III. However, none of the mutants differed significantly from the wild type, although two mutants, 6475 *cmbA*⁻ and 6475 *dnaK*⁻, had a tendency to lose their beneficial effect. The data indicated that none of the genes plays a major role for the protective effect of 6475 on colitis. The protective effect of *L. reuteri* 6475 on the onset and progression of acute colitis in mice has been demonstrated previously (Peña et al., 2004). There are number of studies illustrating the importance of a single gene in association with a physiological function of *L. reuteri*, or its beneficial effect on mucosal integrity. Cell- and mucus-binding protein A (CmbA) is reported to be important in adherence of the bacteria to intestinal epithelial cells and mucus *in vitro* (Jensen et al., 2014). It has been demonstrated that inactivation of any one of the three genes, histidine decarboxylase pyruvoyl type A (*hdcA*), *hdcB* and histidine/histamine antiporter

(*hdcP*), in the histidine decarboxylase gene cluster leads to loss of the TNF-inhibitory activity of *L. reuteri* 6475 (Thomas et al., 2012). Those authors demonstrated in a later study that dihydrofolate synthase (FolC2) is essential for suppression of TNF production by activated human monocytes, and concluded that the gene is important for the anti-inflammatory effect of *L. reuteri* 6475 in a TNBS-induced acute colitis model (Thomas et al., 2016).

However, a recent study showed that inactivation of the LPxTG-anchored amidase improved the anti-inflammatory response and lowered IL-8 expression (Jensen et al., 2015).

Due to the complexity of probiotics-host interactions, it is likely that more genes that play key roles in *L. reuteri* probiosis and contribute to the beneficial effects of *L. reuteri* related to human health and intestinal functionality remain to be discovered. In addition, the combined effect of several genes are perhaps even more likely to have a strong impact on the physiology of the bacteria and its health-related beneficial effects.

5.4 *Lactobacillus reuteri* and mast cell activation and deregulation (Preliminary study)

In the Preliminary study, the effect of *L. reuteri* strains on IgE/antigen-induced degranulation was investigated using murine peritoneal cell-derived mast cells (PCMCs). This choice made it possible to investigate whether probiotics exert their ameliorating effect on inflammation and mucosal integrity, as observed in Papers II and III, by affecting innate immune cells such as mast cells in the mucosa. Mast cells are involved in the immune response and are located in abundance in the lamina propria of the mucosa and also in the submucosal layer. It is known that in intestinal mucosa, mast cells not only respond to antigens, but also play important roles in mucosal barrier function and transport properties (Yu and Perdue, 2001).

There are growing indications that mast cells can mediate some of the beneficial effect of probiotics. Both *in vivo* (Kim et al., 2008, Sawada et al., 2007) and *in vitro* studies (Kawahara, 2010, Kasakura et al., 2009, Cassard et al., 2016) have explored the possible links between probiotic effects and mast cells. However, studies on mast cell contribution in probiosis mechanisms are largely lacking.

The Preliminary study began by evaluating a proper dose of *L. reuteri* and viability of mast cells for the experiment. Based on the evaluation results, 6 hours pre-treatment with 50 MOI of *L. reuteri* was used.

The release of mast cell mediators, including β -hexosaminidase and pro-inflammatory cytokines, was also examined. To determine mast cell

degranulation, a β -hexosaminidase release assay was performed. The activation of mast cells by *L. reuteri* was tested and the results demonstrated that *L. reuteri* did not activate the mast cells except for one out of four strains (6475). In that case, the degranulation was three-fold lower than the stimulatory effect of IgE/antigen in the control. It was speculated that *L. reuteri* 6475 causes exocytosis through lowering the pH of the medium. Several environmental stimuli such as pH, osmolality, radiation and temperature affect this exocytosis (Moon et al., 2014).

The IgE/antigen-mediated stimulation was used for activation of mast cells. Several external stimuli for triggering mast cell degranulation are available and among them, IgE receptor crosslinking has been most notably considered for degranulation (Metcalf et al., 1997). Upon IgE/antigen-dependent stimulation, degranulation and β -hexosaminidase release were observed to be increased approximately 60-fold in untreated cells and *L. reuteri* strains did not exert any inhibitory effect on mast cell degranulation.

Following 6 hours of exposure of mast cells to *L. reuteri*, a low expression of the pro-inflammatory cytokines IL-13 and IL-6 was observed in the unstimulated control. Although mast cells are well known for release of a variety of mediators with different biological activities upon stimulation, expression of pro-inflammatory cytokines such as TNF α , IL-1, IL-5, IL-8, IL-18 and IL-16 has also been reported for unstimulated human mast cells (Lorentz et al., 2000). Consistent with these findings, the Preliminary study also demonstrated expression of IL-13 and IL-6 in unstimulated mast cells (control) and showed that *L. reuteri* treatment did not increase expression of these cytokines.

In addition to the granule-stored mediators (which are stored as preformed granules), mast cells are able to synthesise and release many bioactive compounds, including cytokines and chemokines, upon exposure to specific stimuli (Moon et al., 2014).

The IgE/antigen-dependent stimulation of the mast cells upregulated the expression of IL-13 and IL-6 in untreated cells and *L. reuteri* counteracted this increase intensely, except that the effects of two strains, DSM17938 and R2LC, were not significantly different. Downregulation of mast cell pro-inflammatory cytokines and chemokines have been observed for a number of probiotics and non-pathogenic commensal bacteria. In a study on mast cell-*E. coli* K12 interactions, co-incubation of *E. coli* with human mast cells reduced the Fc ϵ RI-mediated degranulation and downregulated the expression of CCL-4 and CCL-3 (Kulka et al., 2006). A later study observed that *E. coli* DSM 17252 was capable of preventing IgE-induced degranulation in murine mast cells (Magerl et al., 2008). The counteraction of the IgE/antigen mediated degranulation and downregulation of the pro-inflammatory cytokines of murine mast cells such as

IL-4, IL-13 and TNF- α , as well as COX-2 and Fc ϵ RI α in response to heat-killed *L. reuteri* NBRC 15892, has been reported previously (Kawahara, 2010).

The Preliminary study revealed a link between activation of mast cells, degranulation and cytokine upregulation in untreated mast cells. Interestingly, it was found that degranulation of mast cells is not always associated with cytokine release, and a strong decrease in cytokine expression as a result of treatment with *L. reuteri* in the presence of elevated degranulation was observed. In contrast, previous studies have indicated that the release of cytokines can take place without noticeable degranulation, for instance upon exposure to a stimulus such as lipopolysaccharide (LPS) (Galli et al., 2005, Supajatura et al., 2001). In fact, the precise mechanism behind the discriminatory release of mast cell mediators in response to stimuli is not well understood. In particular, the sorting mechanism of cytokines is unknown and little is known about cytokine storage in granules and the pathways involved in initiation of their secretion (Moon et al., 2014). The results of the Preliminary study may suggest that IL-6 and IL-13 are not stored in premade granules like many reported cytokines and chemokines (Okayama et al., 1998) and that release is independent of the classical degranulation pathway, as has been reported for TNF α (Olszewski et al., 2007) and IL-4 (Wilson et al., 2000) (or perhaps similar secretion pathways or exocytosis are not applied for these two cytokines). The mechanisms underlying this complex occurrence and *L. reuteri* cannot be explained easily and need further investigation. However, it can be postulated that 6 hours of exposure is enough time for *L. reuteri* to downregulate new expression of cytokines in response to stimuli, although it failed to inhibit degranulation.

This preliminary investigation should be repeated with additional experiments to confirm the results obtained. These demonstrated that *L. reuteri* can ameliorate the cytokine expression caused by mast cell degranulation in culture environments. The contribution of mast cells to probiosis mechanisms is not known and should be elucidated in future studies. It is possible that *L. reuteri* can ameliorate/dampen inflammation caused by mast cell degranulation in culture environments.

6 Concluding remarks and future perspectives

The overall aim of this thesis was to investigate probiotics-host interactions and, in particular, to study probiosis mechanisms of *L. reuteri* in the context of intestinal permeability and immune modulation in different models. The main findings of this thesis can be summarised as follows:

- The genetic engineering of *L. reuteri* resulted in generation of a mutant, 6475 *dnaK*⁻, and different recombinant stains, including R2LC-CBRluc, 6475-CBRluc-mCherry and R2LC-mCherry. It was demonstrated that the two luminescence and fluorescence reporter genes are suitable markers for studying *L. reuteri* in the complex environment of the GI tract and the data indicated high potential of fluorescence producing bacteria for high-throughput screening of plasmid persistence using flow cytometry. The mCherry- and CBRluc-labelled lactobacilli provide the possibility to study bacterial transit, location and persistence, but they also have significant potential in other *in vivo* and *in vitro* studies. For example, they can be used to discover the fate of bacterial cells during disease conditions when bacterial translocation takes place to the lower layer of the mucosal barrier and to track the bacteria in Peyer's patches, mesenteric lymph nodes and the lymph and blood circulation.
- The effects of four wild type strains of *L. reuteri* (ATCC PTA 6475, R2LC, DSM 17938 and 1563F) were studied in an intestinal infection model. It was demonstrated that *L. reuteri* could dampen the inflammation and counteract the relaxed cell junction permeability. *Lactobacillus reuteri* possibly protects the tight junction region and confers its beneficial effects on permeability through downregulating pro-inflammatory cytokines such as IL-6 and TNF α , and subsequently maintaining and improving expression of the apical junction proteins ZO-1 and E-cadherin. Complementary to the evaluated

targeted molecules, the anti-inflammatory effect of *L. reuteri* should be examined by also evaluating the expression of anti-inflammatory cytokines.

- The tentative roles of five genes in the probiosis mechanism of *L. reuteri* was examined. In *in vitro* and *in vivo* comparative studies, it was demonstrated that the chaperon DnaK, propanediol dehydratase large subunit (PduC), cell- and mucus-binding protein CmbA, sortase and LPxTG anchored amidase may not play major roles in the 6475 probiosis mechanism for attenuating intestinal damage and inflammation.

There is a limited knowledge about genes and factors from *L. reuteri* strains that are directly linked to their beneficial effects and there are relatively few reports on *L. reuteri* mutants that link specific genes to probiosis mechanisms. Investigation of more mutants may help to identify genes involved in *L. reuteri*'s mode of action and, since probiotics-host interactions may be very complex, inactivation of multiple genes could increase the chance to reveal key mechanisms. On the other hand, the results of the *in vitro* study reported here are still preliminary, and there is a need for additional experiments to confirm the findings. For example, the Caco2 cell line could be employed to support the observation on IPEC-J2 and improve understanding of the mechanism of action of *L. reuteri*. In addition, investigating intestinal immunity in other infection and inflammatory models could help to reveal the importance of the targeted genes. The acute colitis model could be replaced by a DSS-induced chronic model of colitis with less severity, which could help to discriminate between wild type and mutants. Therefore, use of a chronic model of colitis is suggested for exploring the probiosis mechanism of *L. reuteri*.

- Studies on the possible association between mast cells and beneficial effects of *L. reuteri* revealed inhibition of pro-inflammatory cytokine expression by *L. reuteri*, while IgE antigen-mediated degranulation still occurred. *Lactobacillus reuteri* strains may confer an anti-inflammatory effect through inhibition of production and release of mast cell cytokines (IL-13 and IL-6) into the extracellular environment, independent of the classical pathway for degranulation (Figure 7).

Lactobacillus reuteri produces lactic acid and lowers the pH, which could affect exocytosis and degranulation, an effect which needs to be considered in future experiments.

Lactobacillus reuteri may activate mast cells in a TLR2-dependent manner, but this needs further investigation. Future experiments also could be designed to study the effect of *L. reuteri* on factors such as spleen tyrosine

kinase (Syk) in signalling cascades which are involved in phosphorylation of signalling molecules and subsequent degranulation.

The study on mast cell-*L. reuteri* interactions was a pilot study and additional experiments need to be conducted in the future. The mechanisms underlying differential mediator release in response to *L. reuteri* are unknown and the mechanisms by which *L. reuteri* influences the mast cell response need to be elucidated in upcoming studies.

There is conclusive evidence for the role of mast cells in the regulation of immunity and mucosal integrity of the gut and mast cells possibly could be considered as one of the potential mediators of the mechanism of action of probiotics.

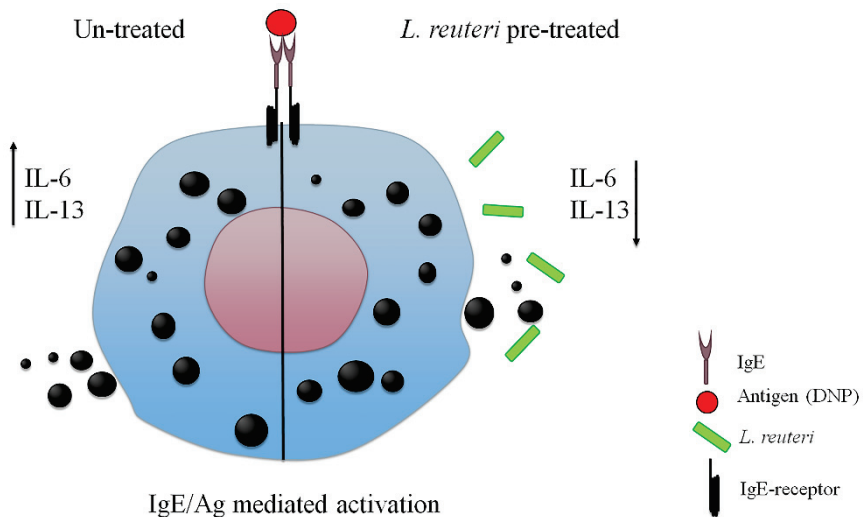


Figure 7. Schematic illustration of the possible effects of *Lactobacillus reuteri* on mast cell degranulation and cytokine release. (Left) IgE/antigen-induced degranulation and cytokine expression and (right) *L. reuteri*-mediated cytokine downregulation.

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Acknowledgements

I must thank to all of those who gave me the possibility to complete this thesis. My PhD study was truly been a special educational scientific journey with many ups and downs which made me a better and stronger person.

Words cannot express all my sincere gratitude to my main supervisor **Dr. Stefan Roos** for his inspiring guidance, continued encouragement, knowledge, patience, advice to me while carrying out this thesis work. I cannot thank you enough for sharing your knowledge with me.

Dr. Hans Jonsson, you proved to be a wonderful supervisor in a difficult situation when a PhD student is encountering pressure and you know how to handle it in the best way. My special thanks to you for your support on my study, official regulations and also your knowledge, patience and advice while I was completing this thesis.

I would like to thank my co-supervisors from University of Uppsala **Prof. Lena Holm** and **Prof. Mia Phillipson** for their knowledge, guidance and support.

My special thanks to my co-supervisor **Dr. Ida Waern** for valuable knowledge and guidance in carrying out the last project of my PhD study.

I'm thankful to **Dr. Sara Wernerssion**, **Dr. Torbjörn Lundh** and **Prof. Eva Hellmen** for valuable discussion and advice and guidance. Many thanks for sharing your scientific knowledge and experience with me.

I would be failing in my duty if I did not state my gratitude for the constant support and encouragement which I received from my true friend **Dr. Bettina Muller**, my special thanks to you.

I owe my sincere thanks to **Anton Pallin** for good discussions and being a good friend to me during the five years of my PhD studies.

I would like to thank **David** and **Evelina** for helping me to perform some of my experiments in your lab.

Anna-Greta Haglund and **Annika Jägare** thank you for helping me in ordering the lab material and reagents and guidance in the lab.

Greta Halting, you are wonderful person, thank you so much for all good advice that you gave me and being there for me in difficult times at the end of my studies.

I would like to thank **Mattias Carlsson, Leticia Pizzul** and **Harald Cederlund, Sara Mehrabi, Mohamad Saberi, Sara Hossieni, Oskar Karlsson lindsjö** and **Hao-yu Liu** for being such good friends to me.

My special thanks to **Magnus Resenquist, Georgios Tzelepis, Emilio Gutiérrez Beltrán** and **Alyona Menina** for sharing scientific knowledge and experience with me.

I would like to thank **all the personnel at the former Microbiology department** for having good chats and fika time together.

Sourena, you are truly an amazing gift and you have always been the source of my joy. My special thanks to you, **my son**.

I would like to thank you **Farzad**, because without you I would be a weaker person.